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This report describes the third year of research effort by a Consortium of investigators who are working to develop, characterize and utilize strains of mice that accurately model tumors that develop in persons with NF1 and NF2. This Consortium has made substantial progress toward accomplishing the goal of generating models of NF1 and NF2-associated tumors for biologic and preclinical therapeutic trials and of exploiting these mice to address biologic and preclinical questions. The Consortium organized a successful conference on the pathologic classification of murine NF-associated neural tumors. Many of the novel strains that have been developed have been shared widely with the research community. The investigators have collaborated closely and have shared expertise and reagents extensively. This NF Consortium has been admitted to the Moue Models of Human Cancer Consortium of the National Cancer Institute and is participating fully in the activities of the group. The current award will support these collaborative studies through 2005.

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INTRODUCTION

Benign and malignant tumors are a major cause of morbidity and mortality in individuals afflicted with NF1 and NF2. The *NF1* and *NF2* genes function as tumor suppressors in humans and in mice. Although a great deal has been learned about the genetics, biochemistry, and cell biology of NF1 and NF2-associated tumors, it has proven difficult to translate these advances into new treatments. The development of accurate, well-characterized mouse models of NF-associated tumors NF1 and NF2 would provide an invaluable resource for bringing improved treatments to NF patients. The overall purpose of this consortium, which is now beginning its fourth year, is to develop such models so that they will serve as permanent resources for the scientific community. These efforts are timely for a number of reasons.

First, recent advances in gene targeting technologies have made it is feasible to introduce many types of alterations into the mouse germline. Indeed, the members of this research consortium developed the initial strains of *Nf1* and *Nf2* mutant mice, which provided major insights into a number of the complications seen in human NF1 and NF2 patients. Since the inception of this consortium effort, we made dramatic progress in improving and extending these models, specifically in the area of engineering conditional mutant alleles of both *Nf1* and *Nf2*. Second, much has been learned about the genetic and biochemical basis of deregulated growth in *NF1* and *NF2*-deficient human cells and in cells from *Nf1* and *Nf2* mutant mice. Genetic analysis of human and murine tumors has provided compelling evidence that *NF1/Nf1* and *NF2/Nf2* function as tumor suppressor genes (TSGs) *in vivo*. Biochemical data have suggested target proteins and pathways for rational drug design. The improved mouse models developed by this consortium now provide an invaluable platform for rigorous preclinical trials of these innovative approaches. Third, new therapies are urgently needed for many of the tumors that arise in individuals with NF1 and NF2. The current treatments for neurofibroma, optic nerve glioma, vestibular schwannoma, and for NF1 and NF2-asociated malignancies are frequently ineffective and carry a substantial risk of long term morbidity. This consortium is highly complimentary to the ongoing efforts to undertake human clinical trials because it will facilitate testing novel agents and approaches in a controlled preclinical setting. The quantity of drug required, expense, and potential liability are all either greatly reduced or eliminated when mouse models are used for preclinical studies. This will facilitate testing a wide range of new therapies that might benefit NF patients. Finally, the Mouse Models of Human Cancer Consortium (MMHCC) of the National Cancer Institute (NCI) is providing a historic opportunity for interactions among 20 research groups that are working to develop, validate, and enhance models of a variety of human cancers. NF is the only inherited cancer predisposition represented within the MMHCC as a discrete disease entity. Our group was admitted to the MMHCC in 2000 and has been participating in its activities. Drs. Jacks, Parada, and Shannon are members of the MMHCC Steering Committee, with Dr. Parada serving as the designated representative of the NF Consortium. Dr. Jacks was Co-Chair of the Steering Committee from its inception until last year, and Dr. Shannon is one of two Co-Chairs currently leading the MMHCC. Thus, this award has provided the NF research community with an exceptional level of representation within the mouse modeling community. The MMHCC is spearheading efforts in areas such as building repositories, devising pathologic classification schemes, imaging mouse tumors, and stimulating interactions with industry in the area of preclinical therapeutics that are of general importance to NF research. The laboratory researchers in this consortium are working closely with the National Neurofibromatosis Foundation (NNFF). This interaction facilitates research in NF1 and NF2

and links basic and clinical researchers with patients. The work initiated under this award is continuing through this renewal award, which commenced in October 2002. We are focusing on the four technical objectives (aims) listed below. Progress in each area is summarized in the following sections.

(1) To enhance existing lines of *Nf1* and *Nf2* mutant mice and to develop new *in vivo* models of NF-associated tumors. We will fully characterize lesions that arise in these mice, focusing on how closely they reproduce the phenotypic, genetic, and biochemical alterations seen in comparable human tumors.

(2) To perform *in vitro* and *in vivo* studies to elucidate biochemical pathways underlying the proliferative advantage of *Nf1* and *Nf2*-deficient cells as a way of identifying molecular targets for therapeutic interventions.

(3) To use these models to perform preclinical trials that will test the efficacy and toxicities of rational therapies for tumors that arise in individuals with NF1 and NF2.

(4) To organize specialized working group meetings that will address: (1) Pathologic Classification of Tumors in NF Mouse Models, and (2) Preclinical Therapeutics in NF Mouse Models, and to develop a pilot program to support preclinical testing of promising treatments in mouse models of NF1 and NF2.

BODY

Background

Tumor Spectrum in NF1 and NF2 Patients. Persons with NF1 are predisposed to benign neurofibromas, optic nerve gliomas, and to specific malignant neoplasms. Individuals with NF1 typically develop multiple neurofibromas that can result in cosmetic, orthopedic, and neurologic disabilities. Optic nerve gliomas are another vexing clinical problem. Although histologically benign, these tumors frequently cause visual impairment or blindness because of their anatomic location. The malignant neoplasms seen in NF1 patients include astrocytoma, malignant peripheral nerve sheath tumor (MPNST), pheochromocytoma, and juvenile myelomonocytic leukemia (JMML). NF2 affects 1 in 40,000 persons worldwide. Individuals with NF2 develop schwannomas along cranial nerves (especially the eighth nerve), as well as peripheral nerves. Other NF2-related tumors include meningiomas, gliomas, and ependymomas.

Production and Characterization of *Nf1* and *Nf2* Mutant Mice. The members of this consortium developed the existing strains of *Nf1* and *Nf2* mutant mice, and have provided these animals to other researchers who are addressing many questions that are relevant to NF1 and NF2 disease. A list of investigators who have received these mice appears at the end of this report. The generation and characterization of these strains has been described in detail in the scientific literature and in our previous progress reports. Briefly, Drs. Jacks and Parada independently disrupted *Nf1* by inserting a neomycin *(neo)* cassette into exon 31 (1, 2). Homozygous *Nf1* mutant ($Nf1^{-/-}$) embryos die *in utero* with cardiac anomalies, which precludes the use of these

mice to study specific aspects of NF1 pathology. To circumvent this problem, Dr. Parada's laboratory harnessed *Cre-loxP* technology to create a conditional *Nf1* allele (3). Importantly, the Parada's lab has shown that the $Nf1^{flox}$ allele functions as a wild-type allele in spite of harboring *loxP* sites and a *neo* gene within its intronic sequences. The $Nf1^{flox}$ allele is readily recombined *in vivo* to make a null allele through co-expression of *Cre* recombinase. Drs. McClatchey, Jacks, and Giovannini used gene targeting to disrupt the *Nf2* locus (4, 5). Homozygous *Nf2* mutant embryos failed earlier than *Nf1-/-* embryos, and failed to initiate gastrulation. Although heterozygous *Nf2* mutant mice are cancer prone, these animals do not develop schwannoma or meningioma. To circumvent the early embryonic-lethal phenotype associated with homozygous inactivation of *Nf2* and to test the hypothesis that the tumor spectrum might be modulated by the rate of the loss of the normal allele in specific tissues, Dr. Giovannini and his colleagues generated a conditional mutant *Nf2* allele (6). A two-step strategy was utilized to construct a mutant $Nf2^{flox2}$ allele characterized by the presence of *loxP* sites in the intronic regions flanking exon 2. As expected, mice homozygous for the $Nf2^{flox2}$ mutant allele ($Nf2^{flox2/flox2}$) were viable and fertile suggesting that the introduction of *loxP* sites did not hamper *Nf2* expression. Induced expression of *Cre* recombinase in $Nf2^{flox2/flox2}$ mice results in biallelic inactivation of *Nf2* in specific tissues. A major research goal of this Consortium is to exploit the conditional mutant alleles of *Nf1* and *Nf2* to develop tractable models of NF-associated tumors for biologic and preclinical applications.

Progress Report

Technical Objective (Aim) 1: To enhance existing lines of Nf1 and Nf2 mutant mice and to develop new in vivo models of NF-associated tumors

An Enhanced Model of JMML. JMML is characterized by over-production of myeloid cells that infiltrate hematopoietic and non-hematopoietic tissues. The hematopoietic system offers a number of advantages as an experimental model including well-defined culture systems to assay the proliferative potential of progenitor cells, techniques that permit adoptive transfer into irradiated recipients, and the ability to perform biochemical assays on primary cells. A hallmark of human JMML cells is that they selectively form excessive numbers of colony forming unit granulocyte-macrophage (CFU-GM) progenitor colonies in methylcellulose cultures exposed to low concentrations of granulocyte-macrophage colony stimulating factor GM-CSF. $Nf1^{-/-}$ fetal hematopoietic cells demonstrate a similar pattern of hypersensitive growth and adoptive transfer of *Nf1*-deficient fetal liver cells into irradiated recipients induces a JMML-like MPD with hyperactive Ras. These mice provide an *in vivo* model for correlating the clinical and biochemical effects of targeted therapeutics on the growth of *Nf1* deficient cells. However, this system is cumbersome and expensive because it requires maintaining a large breeding colony, performing multiple timed matings followed by embryo dissections around E12.5, genotyping by PCR, and injecting fetal liver cells into irradiated hosts.

To circumvent these problems, Dr. Shannon obtained $Nf1^{flox/flox}$ mice from Dr. Parada, performed embryo transfers to move the $Nf1^{flox}$ allele into the UCSF barrier facility, and bred $Nf1^{flox}$ and *Mx1 Cre* mice. In the *Mx1 Cre* strain, Cre recombinase is expressed from the interferon-inducible *Mx1* promoter. *Mx1-Cre,* $Nf1^{flox/flox}$ and $Nf1^{flox/flox}$ littermates received a single injection of pI-pC (500 μg in 50 μL) at 3-5 days of age, and were genotyped at weaning by analyzing DNA prepared from tail clips. As an additional control, some litters that were not

treated with pI-pC were observed for signs of systemic illness. PCR analysis of DNA extracted from peripheral blood leukocytes at 6 weeks of age demonstrated complete excision of exons 31 and 32 in *Mx1-Cre, Nf1*$^{flox/flox}$ mice that received pI-pC (Fig. 1A). Somatic inactivation of *Nf1* was dependent upon inheritance of the *Mx1-Cre* transgene (Fig. 1A). Consistent with previous data, the *Mx1-Cre* transgene was active in other tissues and we detected partial inactivation of *Nf1* in kidney, lung, and other tissues (data not shown).

Cohorts of mice were observed for evidence of disease and by performing serial blood counts. Leukocyte counts and the numbers of differentiated myeloid and lymphoid cells were significantly elevated in the *Mx1-Cre, Nf1*$^{flox/flox}$ animals by 3 months of age (Fig. 1B). Blood smears revealed increased numbers of morphologically normal lymphocytes, monocytes, and neutrophils. Animals with marked leukocytosis showed occasional intermediate myeloid forms (Fig. 1C). Hemoglobin concentrations as well as red blood cell and platelet counts remained within the normal range (data not shown). *Mx1-Cre, Nf1*$^{flox/flox}$ mice developed overt signs of disease beginning between 5 and 6 months of age, which was characterized by hunching, abnormal gait, and a disheveled appearance, and 50% of the animals succumbed by 7.5 months (Fig. 1D). This clinical syndrome was generally not accompanied by dramatic changes in peripheral blood counts, and transformation to acute leukemia did not occur. The development of MPD in *Nf1*$^{flox/flox}$ mice was dependent upon the presence of the *Mx1-Cre* transgene.

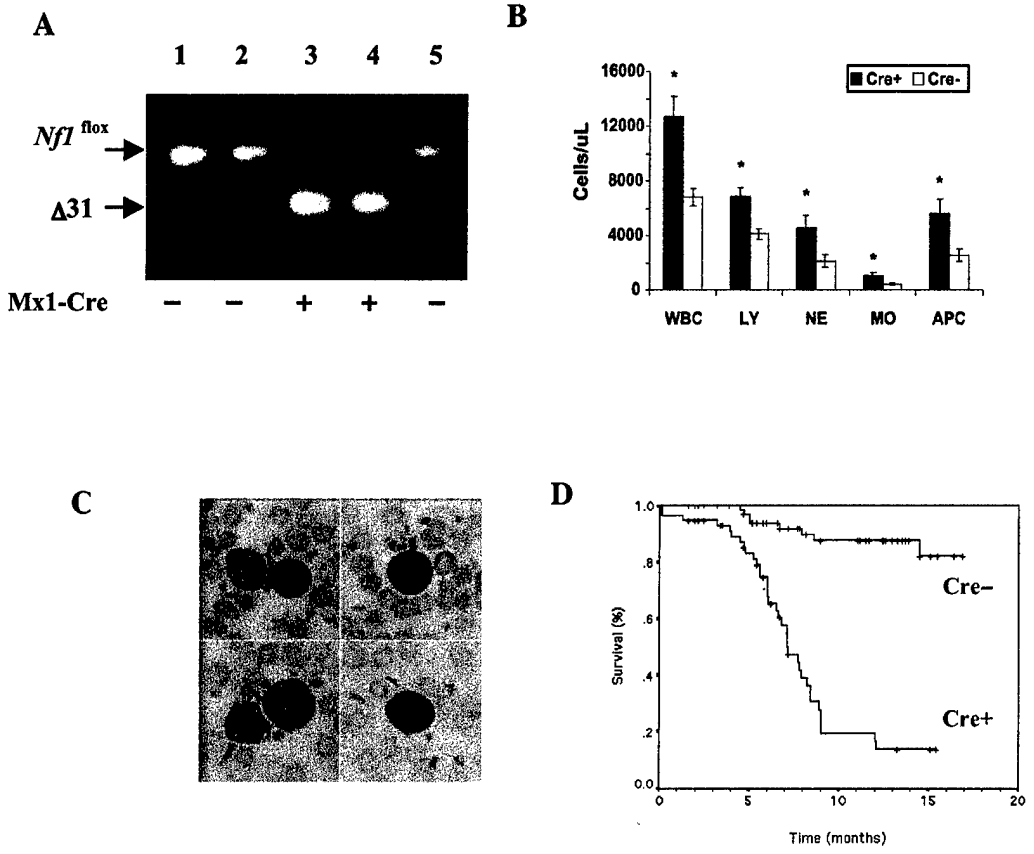


Figure 1 (preceding page). Blood Leukocyte Values and Survival in *Mx1-Cre, Nf1*$^{flox/flox}$ and *Nf1*$^{flox/flox}$ Mice. Panel A. PCR analysis of leukocyte DNA from 6 week old pups that received a single injection of pI-pC shortly after birth. PCR amplification of the unrearranged *Nf1*^{flox} allele yields a 350 basepair (bp) product. A 280 bp fragment corresponding to a deletion of exon 31 (Δ31) is visible in 2 pups that inherited the *Mx1-Cre* transgene ("+") but not in 3 that did not ("-"). Absence of the unrearranged allele in lanes 3 and 4 confirms a high efficiency of somatic recombination. Panel B. White blood cell counts (WBC) in 3 month old pI-pC-treated *Mx1-Cre, Nf1* $^{flox/flox}$ (Cre+) (n = 21) and control *Nf1*$^{flox/flox}$ littermates that did not inherit the *Mx1-Cre* transgene (Cre-) (n = 18). The abbreviations are: LY, lymphocytes; NE, neutrophils; MO, monocytes; APC, absolute phagocyte count (neutrophils + monocytes). Leukocyte counts are expressed ± S.E.M. Asterisks indicate significant differences ($p < 0.05$ by Student's t-test) between the Cre+ and Cre- animals. Panel C. A composite photomicrograph of peripheral blood from a Cre+ mouse shows mature neutrophils (top left), intermediate forms (top right), a monocyte and a mature neutrophil (bottom left), and an intermediate form, which is likely in the monocytic lineage (bottom right). Panel D. Kaplain-Meier analysis demonstrates a significant reduction in survival in Cre+ (n = 59) versus Cre- (n = 72) littermates ($P<0.0001$).

Pathologic analysis of sick *Mx1-Cre, Nf1*$^{flox/flox}$ mice revealed progressive splenomegaly with extensive infiltration of myeloid cells at various stages of maturation (data not shown). There was periportal invasion within the liver, but not in other tissues. The bone marrow was highly cellular and was comprised of myeloid cells at various stages of differentiation. FACS analysis confirmed the presence of a high percentage of myeloid cells (Mac1+ and Gr1+ cells). Increased numbers of Mac-1+, Gr-1^{lo} cells, which are likely to represent immature monocytic cells, were identified consistently. Cytogenetic and spectral karyotype analysis of spleen samples from six diseased mice did not reveal clonal karyotypic abnormalities. According to guidelines published by the Hematopathology Subcommittee of the Mouse Models of Human Cancer Consortium (MMHCC)(7), this disorder is classified as a myeloproliferative disease. This MPD models many features of human JMML including increased numbers of differentiated granulocytic and monocytic cells, hypersensitivity to GM-CSF (see below), and a subacute course. It is similar to the MPD that arises in lethally irradiated mice that are repopulated with homozygous *Nf1*-deficient fetal liver cells(8, 9); however, the course is somewhat more indolent.

JMML bone marrows and *Nf1-/-* fetal liver cells form increased numbers of CFU-GM colonies in the presence of non-saturating concentrations of recombinant murine GM-CSF(8, 10-12). We detected elevated numbers of CFU-GM in the bone marrows of *Mx1-Cre, Nf1*$^{flox/flox}$ mice that were hypersensitive to GM-CSF (Figs. 2A, 2B). In addition, mutant CFU-GM colonies were larger than normal and showed an abnormal spreading morphology (Figs. 2C, 2D). Increased proliferation of myeloid progenitors from *Mx1-Cre, Nf1*$^{flox/flox}$ mice was also reflected by a 2-3 fold expansion in the number of cells recovered from methylcellulose cultures stimulated with 10 ng/mL of recombinant murine GM-CSF. Wright Giemsa staining revealed a higher percentage of monocyte-macrophage cells in *Mx1-Cre, Nf1*$^{flox/flox}$ cultures than in the controls (Figs. 2E, 2F). Consistent with the myeloid infiltration visible in pathologic sections, the spleens of *Mx1-Cre, Nf1*$^{flox/flox}$ mice contained large numbers of CFU-GM, which formed colonies that were similar in size and morphology to those found in the bone marrow. By contrast, no CFU-GM colonies were obtained from the spleens of control mice (data not shown).

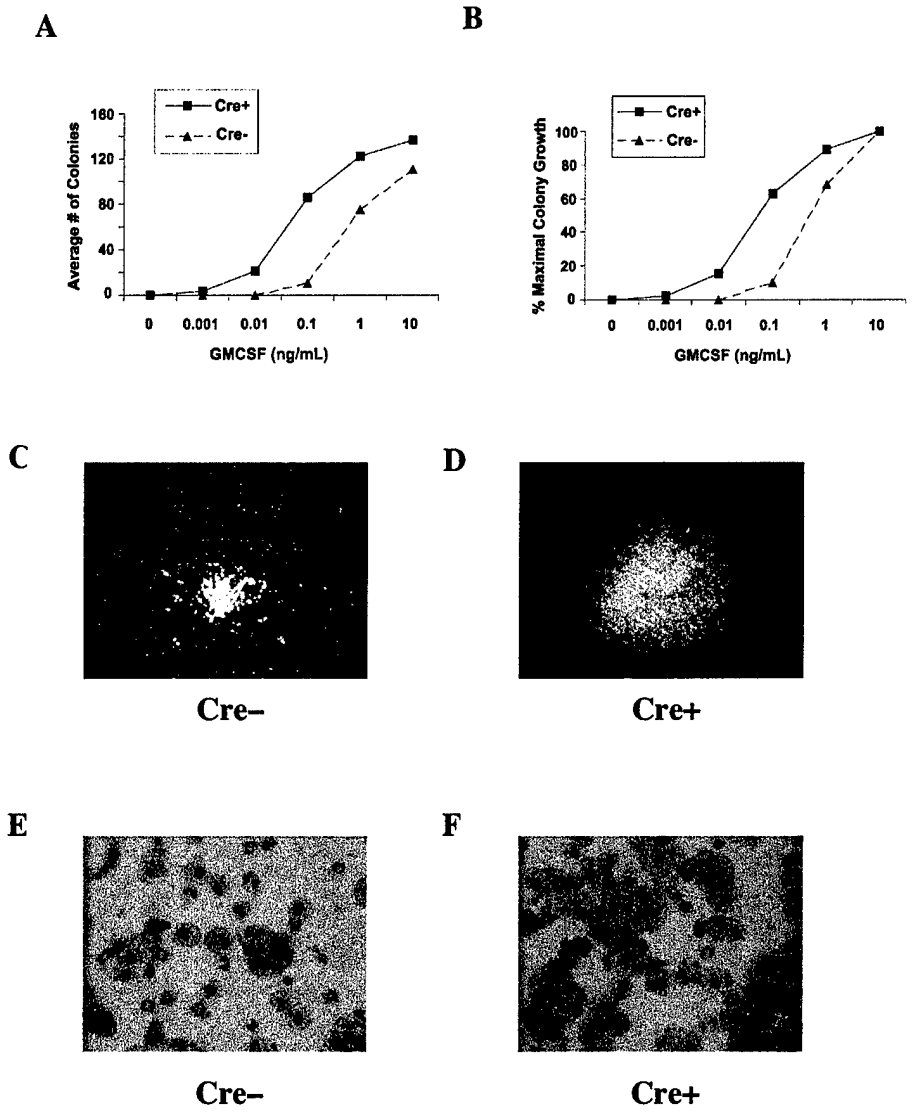


Figure 2. CFU-GM Colony Growth from *Mx1-Cre, Nf1 $^{flox/flox}$* and Cre- Mice. Panels A and B. CFU-GM colony growth at various concentrations of GM-CSF. Bone marrow mononuclear cells were plated in duplicate in methylcellulose. Cre+ bone marrow from mice with MPD show a left shift in the GM-CSF dose-response curve when expressed in terms of total numbers of colonies (panel A) or the calculated percentage of maximal colony growth (panel B). These data are from a representative experiment. Panels C and D. CFU-GM colonies grown from *Mx1-Cre, Nf1 $^{flox/flox}$* and control mice photographed at 40X magnification. A typical CFU-GM morphology from a normal mouse is shown in panel C. The colonies grown from Cre+ mice with MPD are larger and show abnormal spreading (panel D). Panels E and F. Cytospins of CFU-GM colonies stained with Wright-Giemsa from a wild-type mouse (panel E) contain ~70% neutrophils compared to 93% monocyte-macrophage cells in Cre+ mice.

We compared the percentage of Ras in the active GTP-bound conformation and assayed activation of the Ras effectors Akt and MEK in bone marrow mononuclear cells isolated from *Mx1-Cre, Nf1*$^{flox/flox}$ and normal mice. Unstimulated mutant cells showed a modest increase in baseline Ras-GTP levels (Fig. 3A), but phosphorylation of the downstream effectors MEK and Akt were similar in mutant and control bone marrows (Fig. 3B). Exposure to GM-CSF induced robust Ras-GTP, MEK, and Akt activation in both genotypes (Fig. 3B).

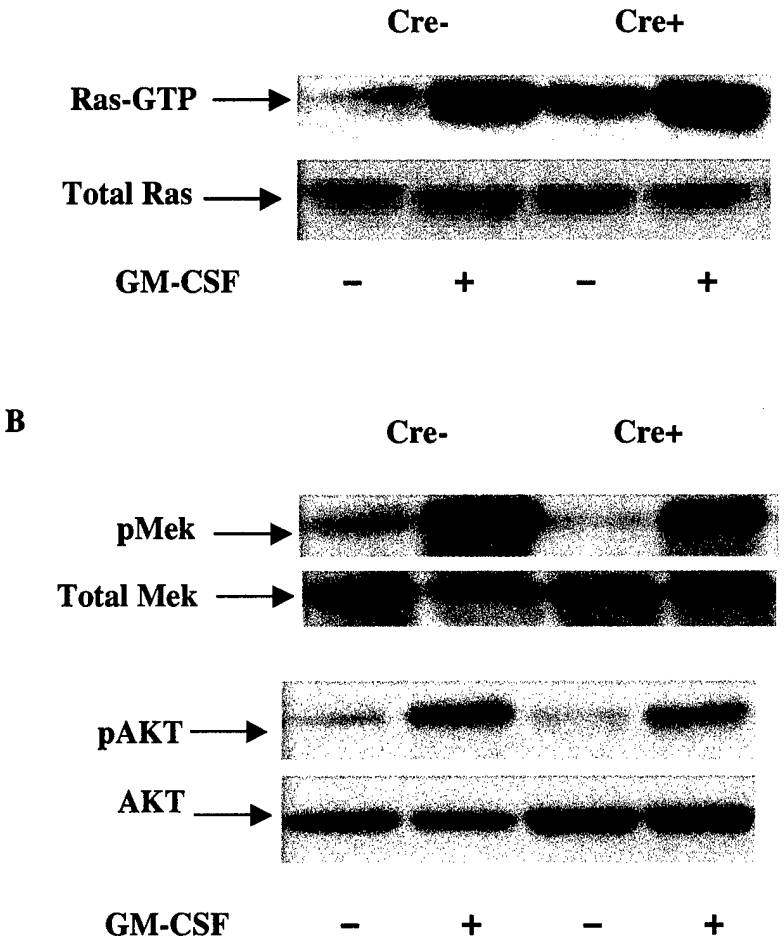


Figure 3. Signal Transduction in Bone Marrow Cells from *Mx1-Cre, Nf1*$^{flox/flox}$ and Cre- Mice. Panel A. Bone marrow cells from mutant and control mice showing Ras-GTP levels. Panel B. Bone marrow cells from mutant and control mice showing basal and growth factor-induced activation of p-Akt and p-MEK in response to GM-CSF.

We measured BrdU incorporation and performed annexin V staining to ascertain the effects of *Nf1* inactivation on the proliferation and survival of primary hematopoietic cells. *Mx1-Cre, Nf1$^{flox/flox}$* mice with MPD and controls were sacrificed 6 hours after a single injection of BrdU, and the percentages of bone marrow and spleen cells that had incorporated the label were determined by flow cytometry. Whereas the fraction of BrdU-positive cells was similar in the bone marrows of diseased and control animals, proliferation was substantially greater within the spleens of *Mx1-Cre, Nf1$^{flox/flox}$* mice (data not shown). Because the percentages of erythroid and myeloid cells in bone marrow and spleen differ markedly between *Mx1-Cre, Nf1$^{flox/flox}$* and control mice, we also assessed BrdU incorporation in Mac1+ cells. Interestingly, proliferation was comparable in bone marrow-derived cells; however, a higher percentage of splenic Mac1+ cells were labeled in the *Mx1-Cre, Nf1$^{flox/flox}$* mice. Apoptosis was assessed by performing flow cytometry on bone marrow cells that had been labeled with an annexin V-green fluorescent protein (GFP). The percentage of freshly isolated cells stained by annexin-V was similar in *Mx1-Cre, Nf1$^{flox/flox}$* and normal mice (data not shown); however, the mutant cells displayed greatly enhanced survival after 24 hours in culture without exogenous growth factors (data not shown).

In summary, we have extensively characterized a robust and tractable new model of JMML by harnessing the *Mx1-Cre* transgene to induce somatic inactivation of *Nf1* in hematopoietic cells. We have also used these *Mx1-Cre, Nf1$^{flox/flox}$* mice to extend our understanding of how loss of *Nf1* perturbs the *in vivo* growth of hematopoietic cells. *Mx1-Cre, Nf1$^{flox/flox}$* mice and cells from these animals can be used to test molecularly targeted agents with pharmacodynamic monitoring of relevant biochemical endpoints in primary target cells. Potential therapeutic strategies for treating JMML include developing GM-CSF receptor antagonists (reviewed in(13-15)), inhibitors of Ras processing enzymes (reviewed in(16)), and agents that interfere with the activation of downstream effectors such as MEK (17). Our biochemical data infer that Ras signaling in primary *Nf1*-deficient cells is perturbed subtly, perhaps because these cells adapt *in vivo*. This may modulate responses to targeted therapeutics. Given the important role of hyperactive Ras in NF1-assocated and sporadic cancers (reviewed in(18, 19)), studies in this tractable disease model might identify agents that have broad therapeutic potential.

Plexiform Neurofibroma Formation in *Krox20 Nf1$^{flox/flox}$* Mutant Mice. The Parada Lab has continued to pursue the novel plexiform neurofibroma models developed in prior fund years. They have generated convincing genetic evidence that the etiology of plexiform neurofibromas requires homozygous inactivation of *Nf1* in Schwann cell precursors (20). However, another surprising outcome of the study has been the equally compelling genetic result that the mutant Schwann cells can only give rise to tumors if the rest of the environment is heterozygous (see Table below). Thus, *Nf1*-deficient Schwann cells in the context of wild type cells cannot give rise to frank tumors. We have commenced investigation into the source of the heterozygous (haploinsufficient) contribution to these tumors. Our first suspect is the mast cell due to their presence in peripheral nerves that contain null, but not heterozygous, Schwann cells. Thus, there appears to exist a unique affinity between these two cell types that could be the catalyst to tumor formation. Current experiments are aimed at determining whether the early appearance of mast cells in a heterozygous environment but not in a homozygous environment is critical for tumor formation. Should this turn out to be the case, detailed studies of mast cell biology will be pursued. It also remains possible that the mast cell presence is just a correlative event. If this is the case, the Parada lab will examine other contributors to tumor formation and using the

conditional knockout mice, systematically examine heterozygous, peripheral nerve sheath cells, fibroblasts, and peripheral nerves for possible active contributions to tumor formation.

Unique Affinity between Nf1-/- Schwann cells and Heterozygous Mutant Mast Cells

Genotype	Schwann cells	Mast cells	Infiltration	Tumors
NF1flox/- or NF1+/-	+/-	+/-	-	-
NF1flox/flox;Kcre+	-/-	+/+	-	-/+
NF1flox/-;Kcre+	***-/-***	***+/-***	***+++***	***+++***

In humans with NF1, MPNSTs appear to invariably evolve from plexiform neurofibromas. Hence the later require the appearance of the former. The Parada lab has preliminary evidence for such a progression in a newly developed mouse model. In this instance, introducing a mutant *p53* allele within the *Krox-20 NF1 flox/-* background leads to neurofibromas that readily progress to malignant tumors. Further preliminary data indicate this process coincides with somatic inactivation of the normal allele of *p53*. Further development of this model will provide novel biologic insights as well as providing a highly relevant system for testing therapeutics.

Generation of Novel Mutant Mouse Strains to Investigate the Specific Role in Development and Tumor Suppression of the Two Major *Nf2* Isoforms. In contrast to the ERM proteins, merlin/schwannomin has two major isoforms (isoform 1 and 2), which differ at their C-terminal end and are conserved in mouse and rat. The two isoforms differ by the inclusion or exclusion of the amino acids encoded by exon 16. The insertion of this 45-bp exon introduces a premature stop codon preventing translation of the last coding exon, exon 17, thereby altering the amino acids at the carboxyl end of the protein. Merlin isoform 1 is a 595-amino acid protein composed of two interacting structural domains, the N- and C-terminus. Merlin isoform 2 (590 amino acids) contains eleven strongly hydrophilic amino acids at its C-terminus. Analysis of the two transcripts during development and in adult tissues has revealed tissue-specific and temporally distinct expression patterns in the rodent, suggesting a functional difference between the encoded proteins that is perhaps mediated by distinct binding partners. Several lines of evidence suggest a correlation between the ability of various natural and mutant merlin isoforms to create intramolecular interactions and the growth suppressive activity of these molecules. In particular, merlin isoform 1, which is the isoform able to inhibit growth when exogenously expressed in rat

schwannoma cells, does contract the intramolecular interaction. On the other hand, merlin isoform 2, which lacks the region encoded by exon 17 required for proper folding, does not affect schwannoma cells growth. Dr. Giovannini has used a genetic approach to develop mouse models to study the specific roles of *Nf2* isoform 1 and 2. Two new mutant *Nf2* alleles were engineered: the *Nf2*iso1- allele in which exon 16 is fused to exon 15 (knock-in ex 16) and the *Nf2*iso2- allele that lacks exon 16 (knock-out ex 16). In contrast to *Nf2*-/- mice that die early during development, mice lacking isoform 1 (*Nf2*iso1-/-) or 2 (*Nf2*iso2-/-) are viable and physically normal suggesting that the role of Nf2 in development is not isoform-dependent. The embryonic lethality of *Nf2* null mice was also rescued when the copy number of the remaining isoform was reduced to the heterozygous state (*Nf2*iso1+/-; iso2-/- and *Nf2*iso1-/-; iso2+/-). There mice are now used for studies on the effects of *Nf2* isoform deficiencies on cancer susceptibility and Schwann cell proliferation.

Use of Avian Leukosis and Sarcoma Virus to Target Mature Schwann Cells *In Vivo*. Dr. McClatchey has generated transgenic mice that express the avian leukosis and sarcoma virus type a (tv-a) receptor under the control of the P0 promoter. Expression of tv-a renders murine cells susceptible to infection by recombinant avian retroviruses that have been engineered to express many genes of interest, including *Cre*. Such recombinant viruses have been shown to replicate to a very high titer, allowing for efficient infection *in vitro* and *in vivo*. Initially, optimal conditions for expression of a lacZ reporter in mature Schwann cells will be ascertained by introducing lacZ-expressing avian retroviruses into a site adjacent to the sciatic nerve with and without nerve transection to induce Wallerian degeneration and proliferation of mature Schwann cells. These experimental conditions are being employed to infect mature Schwann cells with a Cre-expressing virus. This strategy has the added advantage of producing focal loss of *Nf2*, thus more closely mirroring the context of schwannoma development in humans. Dr. Sandra Orsulic, who helped to develop this technology in the laboratory of Dr. Harold Varmus, is assisting Dr. McClatchey with these studies.

Diffuse Ependymal Cell Hyperproliferation and "Mini" Ependymomas in the Nestin-Cre; $Nf2^{2lox/1lox}$ Mouse Model. Pediatric primary brain tumors are the second most frequent malignancy of childhood and are now the leading cause of death from childhood cancer, accounting for approximately 24% of cancer-related deaths. Ependymoma, a type of primary brain tumor that arises from the specialized glial cells (ependymal cells) that line the ventricular system, may develop at any age. However, ependymomas are particularly common in young children, accounting for 6-12% of pediatric primary brain tumors, and this tumor type occurs at increased frequency in children with NF2. Although ependymomas are slow growing and histologically classified as WHO grade II/IV, the 5-year progression free survival is only 50%, with children under two years of age having a particularly dismal prognosis. Since mutation of the *NF2* gene is the only well documented genetic alteration in human ependymomas, the Jacks laboratory is generating mouse models of ependymoma in which the initiating event is loss of *Nf2*. Previous work from the Jacks lab has shown that $Nf2^{-/-}$ mice die in early embryogenesis secondary to defects in the extraembryonic tissues, and that $Nf2^{+/-}$ mice develop malignant tumors but not the tumor types characteristic of the human disease (schwannoma, meningioma, and ependymoma). Therefore, all of the mouse models now under construction will make use of a conditional *Nf2* allele ($Nf2^{2lox}$) in which exon 2 have been flanked by LoxP sites. Produced in Dr. Giovaninni's laboratory, the $Nf2^{2lox}$ allele behaves like the wild-type allele until it is exposed

to Cre recombinase. Exposure to Cre leads to deletion of the DNA between the LoxP sites, forming the $Nf2^{1lox}$ allele that behaves like the null. The use of the $Nf2^{2lox}$ allele provides more control over the cell types that lose *Nf2* and the timing of loss, thereby increasing the chances of avoiding early lethality and unwanted malignant tumors.

At this time, no ependymal cell-specific promoter has been characterized. Such a promoter would be the ideal way to drive Cre expression in ependymal cells. In the absence of such a promoter, the Jacks laboratory is using Cre transgenic mice in which Cre is expressed within ependymal cells but also within other cells of the body and/or brain. Like neurons and the other glial cells that form the brain, ependymal cells develop from neuroepithelial cells, which express the intermediate filament nestin. Neuroepithelial cells are thought to give rise to radial glial cells, which in turn give rise to immature ependymal cells (tanycytes). Tanycytes express nestin and another intermediate filament glial fibrillary acidic protein (GFAP). Tanycytes mature into ependymal cells that continue to express nestin but lose GFAP expression. A few tanycytes remain present in adult animals. Both Nestin-Cre[5] and GFAP-Cre (Housman laboratory, MIT; unpublished data) transgenic mice are currently available. By crossing Nestin-Cre mice to mice containing a Cre reporter (Rosa26-LSL-LacZ), the Jacks lab has confirmed that the Nestin-Cre mice do express Cre within ependymal cells. Similarly, by performing immunohistochemistry against Cre, they have shown that the GFAP-Cre mice also express Cre within a small subset of ependymal cells, likely tanycytes. In addition to ependymal cells, Nestin-Cre is highly expressed in neurons and other glial cells in the brain and throughout most tissues of the body. The Jacks laboratory is still in the process of characterizing the expression pattern of GFAP-Cre.

As a first step toward generating a mouse model of ependymoma, the Jacks laboratory has crossed Nestin-Cre mice to $Nf2^{2lox}$ mice. Because the Nestin-Cre transgene inserted into an imprinted locus, mice that inherit paternally derived Nestin-Cre (Nes-Cre^{p}) lose *Nf2* in virtually all cells within the brain and are mosaic for *Nf2* loss in the body. Mice that inherit maternally derived Nestin-Cre (Nes-Cre^{m}) are mosaic for *Nf2* loss in the brain and body. Nes-Cre^{p}; $Nf2^{2lox/1lox}$ mice exhibit a wide range of developmental abnormalities. The majority of these mice die around the time of birth. Approximately 26% have neural tube defects. In those mice where the neural tube closed, they observe diffuse hyperproliferation of ependymal cells around the ventricles and central canal of the spinal cord. Immunohistochemistry for the proliferation marker, Ki-67, showed a significant increase in the number of cycling cells in the ependyma of mutants compared to controls (3.28 positive cells/100mm vs. 0.29 positive cells/100mm, P=0.07 by f-test). Immunohistochemistry for the apoptosis marker, cleaved caspase 3, showed very low levels of apoptosis in the ependyma of both mutants and controls. Thus, loss of *Nf2* alone appears to be sufficient to cause an increase in ependymal cell number.

The majority of Nes-Cre^{m}; $Nf2^{2lox/1lox}$ mice also die around the time of birth. However, a few survive 1-2 weeks into the postnatal period, although they are severely runted. Examination of the brains of the longest living Nes-Cre^{m}; $Nf2^{2lox/1lox}$ mice revealed focal areas of ependymal cell hyperproliferation. In one lesion arising from the wall of the third ventricle, the ependymal cells formed tubules resembling the "true" rosettes of human ependymomas. Dr. Jacks believes these focal lesions may represent the earliest stage of ependymoma development and has termed these lesions "mini" ependymomas.

Although the Jacks lab are seeing diffuse ependymal cell hyperproliferation and "mini" ependymomas in Nes-Cre^{m}; $Nf2^{2lox/1lox}$ mice, this model is of limited use because of the early lethality and the numerous associated developmental abnormalities. To circumvent these problems, they has crossed the GFAP-Cre transgenic mice to $Nf2^{2lox}$ mice. They anticipate that

GFAP-Cre will be expressed at a later stage of development than Nestin-Cre and will affect a narrower range of cell types. At this point, they have generated several GFAP-Cre; $Nf2^{2lox/1lox}$ mice that appear healthy at 4 weeks after birth. They are closely monitoring these animals for hydrocephalus and behavioral abnormalities that might indicate the presence of a brain mass.

Construction of Drug-Inducible *Cre* Mice to Locally and Temporally Control Deletion of the *Nf1* and *Nf2* genes. In addition to using conventional transgenic Cre lines, the Jacks laboratory is generating drug-inducible Cre mice that will permit local and temporal control of somatic *Nf1* or *Nf2* gene deletion. They have inserted a modified Cre, $CreER^{T2}$ (Cre fused to an altered human estrogen receptor that is activated by the drug tamoxifen)[7], downstream of the ubiquitous Rosa26 promoter. $CreER^{T2}$ is ten times more sensitive to tamoxifen than the first generation of CreER constructs, therefore it will avoid confounding issues related to tamoxifen toxicity. The Jacks laboratory has demonstrated that the $CreER^{T2}$ construct is functional in ES cells, and has generated mice that have $CreER^{T2}$ in the germline. By crossing the Rosa26-$CreER^{T2}$ mice to Protamine-Cre mice, they are in the process of deleting the puromycin selection cassette. Once the selection cassette is removed, they will cross the Rosa26-$CreER^{T2}$ mice to mice containing a Cre reporter (Rosa26-LSL-$LacZ^{2lox}$), and analyze the pattern of Cre expression with and without administration of tamoxifen. To generate ependymomas, they plan to steriotactically inject tamoxifen into the ventricular space, thereby preferentially activating Cre in the ependymal cells that line this space. Since they can control the timing of injection, they will know precisely when tumor formation is initiated and can then follow tumor progression. In collaboration with the Housman lab (MIT), the Jacks laboratory is in the process of generating a GFAP-Cre mER^{T2} mouse that will allow for greater cell type specificity as well as temporal control. These strains will also be useful in tissue and temporal control of deletion of *Nf1* in collaboration with Dr. Parada and Dr. Shannon in the development of improved models of neurofibroma, astrocytoma, and myeloid leukemia.

Technical Objective (Aim) 2: To perform in vitro and in vivo experiments that will elucidate molecular targets for therapeutic interventions.

Background

In addition to generating mouse models for testing potential therapeutic agents for NF1 and NF2-associated tumors, the identification and validation of molecular targets remains a significant rate-limiting step in the discovery of effective therapies for these diseases. Considerable work from our laboratories and others has demonstrated that loss of *NF1/Nf1* function leads to hyperactivation of the Ras signaling pathway.

Post-translational processing of Ras proteins has attracted considerable interest as a potential target for anticancer drug discovery. Ras proteins undergo posttranslational modification at a common C-terminal *CAAX* sequence (reviewed in (21)). Processing is initiated by farnesyltransferase (Ftase), which attaches a farnesyl lipid to the thiol group of the cysteine (the "C" of the CAAX motif). Prenylation targets Ras to membranes, and is required for the biologic activity of normal and oncogenic Ras. Ftase inhibitors have shown promise as anticancer agents (21) and are currently being tested in NF1 patients with plexiform neurofibroma. However, K-Ras and N-Ras are substrates for geranylgeranyltransferase 1 (GGTase 1) and are processed by this alternative pathway when Ftase is inhibited. Indeed,

extensive data now support the view that non-Ras C*AAX* proteins are critical *in vivo* targets of the Ftase inhibitors (reviewed in (16)). After prenylation, the carboyxl terminal three amino acids are released by Rce1, an integral membrane endoprotease of the endoplasmic reticulum. The final step in Ras processing involves methylation of the prenylcysteine by isoprenylcysteine carboxyl methyltransferase.

Agents that interfere with various signaling pathways downstream of Ras (such as the Raf1-MEK-MAPK and phosphoinositide-3-OH kinase (PI3K)-protein kinase B (PKB; also known as Akt) cascades) are of obvious interest in the treatment of NF1-associated tumors. Upstream receptor tyrosine kinases and their ligands may also be required for the growth of specific *NF1/Nf1* mutant tumors, including GM-CSF in the case of myeloid leukemia and epidermal growth factor receptor for MPNSTs (22). It is important to note that genetic experiments in *Drosophila* have also demonstrated link between loss of neurofibromin function and PKA signaling.

Studies by the members of this consortium and others are beginning to elucidate the effects of *NF2/Nf2* mutations on cellular signaling cascades. Our recent progress in this area is described here. As summarized in the following sections, we are continuing to exploit a combination of genetic, biochemical, and cell biologic experiments to uncover genes that cooperate in generating NF-associated tumors and to characterize proteins that might be targets for therapeutic intervention.

Rce1 x *Nf1* Cross and Analysis of Cellular and Biochemical Phenotypes. As the only known *CAAX* protease in mammalian cells, Rce1 represents an attractive drug target for treating disorders associated with hyperactive Ras such as NF1. The murine *Rce1* gene was disrupted by Dr. Stephen Young to elucidate its role in development and tumorigenesis. Genetic ablation eliminates Ras endoproteolytic activity, which results in mislocalization of ~50% of Ras away from the plasma membrane. Importantly, *Rce1*-deficient cells are unable to process either farnesylated or geranylgernaylated substrates. Homozygous mutant embryos *(Rce1-/-)* demonstrate late embryonic lethality with normal organogenesis (23). Dr. Shannon's laboratory performed adoptive transfer, biochemical, and competitive repopulation experiments to define the importance of *Rce1* in the growth of hematopoietic cells. These data were published recently (see attached paper by Aiyigari). Briefly, *Rce1-/-* fetal liver cells efficiently restored hematopoiesis in irradiated recipient mice. Although there was some variability between individual experiments, colony growth was similar for wild-type, *Rce1+/-, Rce1-/-* cells. ERK kinase activities were measured in bone marrow collected 3–6 months after adoptive transfer. In multiple experiments, wild-type and *Rce1-/-* cells demonstrated equivalent basal and GM-CSF-stimulated ERK kinase activities. Wild-type and *Rce1-/-* fetal liver tester cells were injected into irradiated hosts with the same reference population of BoyJ competitor cells to directly compare their repopulating potentials. Cells of both genotypes demonstrated equivalent repopulating potentials over a dose range that produced 10 - 70% donor cell chimerism (data not shown). These data provide strong evidence that inactivation of *Rce1* does not impair the proliferative capacity of normal hematopoietic cells.

Ongoing Studies of Ras Processing and Upstream Adapter Molecules as Therapeutic Targets in NF1 Disease. Based on our findings in *Rce1* mice, we have not begun crossing the conditional $Nf1^{flox}$ allele onto the heterozygous *Rce1*mutant background. Instead, we plan to take advantage of a new model of aggressive MPD developed in the Shannon lab by inducing expression of a

mutant *Kras* allele engineered by the Jacks lab in hematopoietic cells. Furthermore, Dr. Young has recently developed conditional mutant alleles of both *Rce1* and of the Ras methyltransferase *Icmt*. We can assess the effects of these alleles on the MPD phenotype in a single generation cross onto the *Kras* background. These studies, which will allow us to assess if inhibiting Ras processing represents a viable therapeutic strategy, are underway. If the results in the *Kras* background demonstrate attenuation of this aggressive MPD, we will determine if somatic inactivation of *Rce1* and/or *Icmt* also alters the phenotype of the MPD that results from inactivation of *Nf1*. Gab2 is a adapter molecule that links activated hematopoietic growth factor receptors to Ras. Although *Gab2* mutant mice are normal, the ability of the *BCR-ABL* oncogene to induce MPD was recently shown to be Gab2-dependent (24). Since signaling from the GM-CSF receptor plays a central role in the MPD that results from *Nf1* inactivation (9), we reasoned that Gab2 might similarly be required to induce leukemia in *Nf1* mice. The Shannon lab has initiated a cross between *Mx1-Cre, Nf1*$^{flox/flox}$ and *Gab2* mutant mice to test this hypothesis. If these studies reveal a requirement for Gab2 in disease pathogenesis, this would identify a new class of potential therapeutic targets upstream of Ras and neurofibromin.

Growth Control in *Nf1/p53* Tumor Cell Lines. The Parada lab has continued to investigate *Nf1/p53cis* MPNST cell lines. These studies have provided several lines of evidence that aberrant activation of Ras is important in the maintaining the malignant phenotype over time. The initial experiments to inhibit growth potential and tumorigeneicity were performed with early passage cells. Recent experiments showed that the growth of late passage tumor cells can also be inhibited by blocking the Ras pathway. The Parada lab is continuing to pursue this area this issue and has begun to comparing the growth of these cells with cell lines derived from MPNSTs that have arisen within the context of neurofibromas. Both comparative genomic as well as cell biologiclal experiments will be performed to assess the similarities and potential differences between the two model systems. In the meantime, Exelixis and other biothechnology companies are proceeding with the licensing these cell lines for use in initial high throughput drug screens.

Progesterone Receptor Expression in Neurofibromas. Neurofibromas are benign tumors of the peripheral nerve sheath, which have several interesting properties. First, they occur both sporadically and in association with NF1. Second, neurofibromas invariably contain a mixture of cell types found in normal peripheral nerves (axonal processes of neurons, Schwann cells, perineurial cells, fibroblasts, and mast cells), suggesting that paracrine interactions between different cell types may influence tumor growth. Third, there are intriguing links between the growth of neurofibromas and levels of circulating hormones: neurofibromas often first appear around the time of puberty, increase in number and size during pregnancy, and shrink after giving birth, indicating that blood-born signals, such as hormones, may promote tumor growth.

As a first step toward identifying the critical hormone(s) that regulates neurofibroma growth, the Jacks laboratory examined 59 human neurofibromas for the expression of estrogen receptor (ER) and progesterone receptor (PR), as estrogen and progesterone were attractive candidate hormones. The majority (75%) of neurofibromas expressed PR, whereas only a minority (5%) of neurofibromas expressed ER. PR expression was rarely seen in other peripheral nerve sheath tumors (schwannomas and malignant peripheral nerve sheath tumors) and was not detected in normal peripheral nerve (25). Within the PR-positive neurofibromas not every cell expresses PR. This is to be expected given the cellular heterogeneity of neurofibromas. By

double immunofluorescence, they demonstrated that PR was expressed in the non-neoplastic tumor-associated cells and not by the neoplastic Schwann cells. To affect tumor growth, the PR-expressing cells may produce paracrine factors that increase the proliferation and/or survival of the neoplastic Schwann cells.

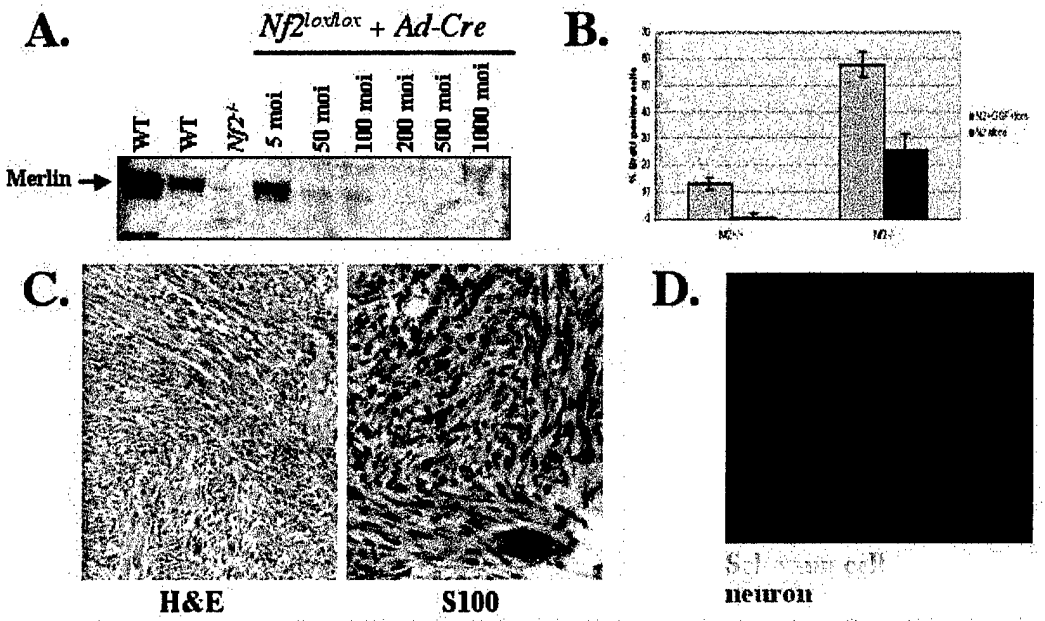
Currently, surgical resection is the only means of treating neurofibromas. However, complete surgical resection is not always possible for large lesions or for NF1 patients who may have hundreds to thousands of lesions. Furthermore, surgical resection of intraneural neurofibromas requires sacrifice of the parent nerve leading to significant neurological deficits. Based on our results, we propose that antiprogestins, such as Mifepristone (RU486), may be useful for the treatment of neurofibromas as an alternative to surgery, to reduce the size of lesions so that smaller surgical procedures could be performed, and/or to slow malignant progression of the plexiform subtype of neurofibromas.

Future work on this project will proceed along several lines. In collaboration with clinicians in the Partners Healthcare System, the Jacks laboratory hopes to initiate a clinical trial to test the hypothesis that antiprogestins can slow neurofibroma growth. The role of progesterone in regulating neurofibroma growth will also be examined by crossing existing mouse models of neurofibromas to mice with homozygous deletions in the gene encoding PR. They also plan to use the mouse models of neurofibromas to test the efficacy of antiprogestins. One would predict that either eliminating PR or administering antiprogestins should slow neurofibroma growth. If the mouse model system is sufficiently similar to the human situation, it may be possible to use mouse nerve culture systems to dissect out the critical paracrine interactions mediated by PR.

Growth and Survival of *Nf2*$^{-/-}$ Schwann Cells. Schwann cells play a central role in the pathologic complications of NF2. It is therefore essential to rigorously interrogate the consequences of *NF2*-deficiency in this cell type. In other ongoing studies, Dr. McClatchey's laboratory has recently identified several 'signatures' of *Nf2*-deficiency in primary murine cells of other types (26). Specifically, they have identified a key role for merlin in mediating contact-dependent inhibition of proliferation through the formation of cadherin-mediated adherens junctions (AJs). Their results suggest that merlin is required for stabilizing the interaction between nascent AJs and the actin cytoskeleton. They have also found that *Nf2*$^{-/-}$ cells of several types, including cardiomyocytes and osteoblasts undergo abnormal programs of differentiation (unpublished results). Dr. McClatchey's laboratory has now generated primary Schwann cell cultures from the conditional *Nf2*-mutant mice generated by Dr. Giovannini and asked whether there are also 'signatures' of *Nf2*-deficiency in Schwann cells.

Primary Schwann cells were isolated from dorsal root ganglia (DRG) of E12.5 embryos or the sciatic nerves of 3-week-old mice. The purity of the cultures was assessed by protein S-100 immunocytochemistry. The cultures were infected with experimentally determined titers of empty vector or *Ad-Cre* to inactivate *Nf2*. Recombination of the conditional allele was confirmed by PCR and loss of merlin was confirmed by Western blot analysis. *Nf2*-deficient Schwann cells isolated from the E12.5 DRG exhibited a marked growth advantage and like *Nf2*$^{-/-}$ fibroblasts and osteoblasts, did not undergo contact-dependent inhibition of proliferation (Figure 4; and data not shown). Importantly, *Nf2*$^{-/-}$ Schwann cells also proliferated well without exogenous glial growth factor (GGF); in contrast, wild-type Schwann cells were completely dependent upon GGF for proliferation (27) (Figure 4). This is consistent with the growth-factor independence displayed by *Nf2*$^{-/-}$ fibroblasts (26). Moreover, when injected subcutaneously into nude mice, *Nf2*$^{-/-}$ Schwann cells frequently formed tumors (6/8), suggesting that few, if any

additional genetic events are required for their transformation (Figure 4). Importantly, when isolated from the sciatic nerve of 3-week old mice, $Nf2^{-/-}$ Schwann cells do *not* exhibit a growth advantage relative to wild-type, suggesting that either the timing or anatomical location is a critical factor in determining the sensitivity of Schwann cells to *Nf2*-deficiency. The *P0-tva* mice described above represent a valuable tool for defining the temporal and spatial variability of Schwann cells to *Nf2*-deficiency.



Fig, 4 Generation and evaluation of E12.5 DRG-derived $Nf2^{-/-}$ Schwann cells. A) Western blot analysis of merlin levels in $Nf2^{lox/lox}$ Schwann cells infected with increasing amounts of *Ad-Cre*. MOI; multiplicity of infection. B) $Nf2^{-/-}$ Schwann cells exhibit a distinct proliferative advantage in the presence (N2+GGF+fors) and absence (N2 alone) of growth factors. Cells were labeled with bromodeoxyuridine (BrdU) and analyzed by immunofluorescence microscopy. C) Subcutaneous injection of $Nf2^{-/-}$ Schwann cells frequently (6/8) resulted in the formation of tumors that were immunoreactive for the S100 Schwann cell antigen. D) Establishment of Schwann cell:neuron coculture system for studying the myelinating potential of $Nf2^{-/-}$ Schwann cells.

Dr. Giovannini's laboratory has devised a simple method to purify mitotically active Schwann cells (SC) from peripheral nerves of adult mice. Nerves are predegenerated *in vitro* for 7 days and after dissociation cells are plated on poly-L-lysine/laminin coated dishes in N2 serum-free culture medium supplemented with forskolin and heregulin-ß1. Primary cultures are purified from contaminating fibroblasts by magnetic cell sorting (MACS) based on SC membrane specific expression of $p75^{NGFR}$ and enriched to about 99% of SC after MACS from 34% to 91% before sorting. After sorting, purified wild-type adult mouse SC can be propagated for 3 passages until confluent to a total surface of 160 cm^2/mouse (2 sciatic and 2 trigeminal nerves). In addition, they have shown that this method can be used to purify tumoral SC from mouse NF2 and NF1-related schwannomas and neurofibromas. The extension of the procedure to the purification of neural crest derived tumors provides an additional tool for the study of the genetical and biochemical changes that occur during NF-related tumor development.

Merlin is an Inhibitor of the p21-Activated Kinase Pak1. Based on the observations from Dr. McClatchey that merlin inhibits Rac-signaling at some level, the Jacks laboratory has assessed the possibility of a stable interaction between merlin and Pak kinases, known effectors of Rac signaling. NIH3T3 cells were cotransfected with expression vectors for merlin and Pak1 and association of the proteins was demonstrated by co-immunoprecipitations. To assess the interaction in an additional cell type, a Rat scwhannoma cell line (RT4-DP6) was examined. These cells express relatively low levels of endogenous merlin and detectable endogenous levels of Pak1. As in the case of NIH3T3 cells, association of the proteins was demonstrated by co-immunoprecipitation. To determine if merlin and Pak can interact directly, the association of the two proteins was confirmed *in vitro* using bacterially-expressed or *in vitro* translated proteins. To further delineate the regions of Pak1 that mediate interaction with merlin, the Jacks laboratory has assessed the binding of different merlin domains to full length Pak1 *in vitro*. The N-terminal FERM domain (1-313) and the C-terminal tail of merlin (314-595) were transcribed and translated *in vitro* and tested for their ability to interact with GST-Pak1. While the N-terminal FERM domain interacted efficiently with GST-Pak1, the C-terminal fragment failed to interact. Trying to further narrow down the interacting domains in the merlin FERM, they tested the ability of either the F1-F2 domain (1-217) or the F3 domain (218-313) to bind to Pak1. Both domains interacted well with the kinase, indicating that there are multiple binding sites in the FERM domain, involved in the interaction of merlin with Pak1. They next tested the possibility that the FERM domain could bind to the N-terminal regulatory domain of Pak1 (70-143), which contains the cdc42/Rac-binding domain (PBD). The FERM domain interacts with the PBD, although the interaction was weaker than the interaction with full-length Pak1.

The Jacks laboratory has gone on to demonstrate that the interaction of merlin and Pak1 is dynamic and influenced by cellular adhesion and cell density. Specifically, the interaction of merlin and Pak was enhanced under conditions demonstrated to be inhibitory to Pak activation in NIH3T3 cells and in the RT4-67 Rat schwannoma cells.

As merlin bound the Pak1 PBD domain, they tested the possibility that merlin can inhibit the interaction between Rac and Pak1. RT4-67 cells were grown in the presence or absence of doxycycline and Pak1-Rac interaction was examined by immnuoprecipitations. Overexpression of merlin inhibited the interaction between Pak1 and Rac, as demonstrated by the reduced levels of Rac co-immunoprecipitated with Pak1 and vice versa. The overall reduction in this interaction was approximately 4- fold in the presence of merlin. While levels of Rac1 were not altered in the presence or absence of merlin, the levels of the GTP-bound form of Rac1 were decreased by approximately 2-fold in cells expressing merlin. Based on these observations, they propose that merlin can inhibit Pak1 activation by binding directly to the Pak1 PBD and interfere with the binding of active-Rac to the Pak1 PBD. Merlin might also function by directly reducing the levels of active-Rac1 that is available to bind and activate Pak1. Similarly, merlin could interfere directly with the interaction of Pak1 to paxillin or this effect could be a consequence in the reduction of active Rac, which is required for the recruitment of Pak1 to focal adhesion complexes.

They next tested the possibility that merlin expression might affect Pak1 activation. The phosphorylation status of Pak1 serves as a direct indication of the activation status of the kinase. To examine differences in the phosphorylation state of Pak1, the Jacks laboratory used 2-dimensional gel analysis to separate the different forms of activated Pak1. To address the effect of merlin on Pak1 activation *in vivo*, the consequence of loss of merlin expression in MEFs was examined. Mouse embryo fibroblasts (MEFs) were prepared from animals carrying a conditional

knockout (floxed) allele of *Nf2* ($Nf2^{flox2}$) (6). In addition to the $Nf2^{flox2}$ allele, the cells either carried a wild-type *Nf2* allele ($Nf2^{flox2}$/+) or a *Nf2* deletion allele ($Nf2^{flox2}$/-)(28). The MEFs were then infected with adenovirus expressing Cre-recombinase (ad-Cre), which led to the inactivation of the floxed *Nf2* allele. To test for loss of merlin expression in the ad-Cre treated $Nf2^{flox2}$/- MEFs, extracts were prepared 96 hours after infection. One milligram of extract was used to immunoprecipitate merlin and merlin levels were examined by western blotting. The ad-Cre treated $Nf2^{flox2}$/- MEFs lacked detectable merlin protein, 96 hours after infection, while merlin levels were not altered in ad-Cre $Nf2^{flox2}$/+ MEFs. As a control, infection of the cells with an adenovirus expressing the LacZ gene did not alter merlin levels (not shown). The status of Pak1 was then analyzed in the MEFs. The ad-Cre-treated and adenovirus control-treated $Nf2^{flox2}$/- and ad-Cre treated $Nf2^{flox2}$/+ MEFs were plated at the same cellular densities and allowed to adhere to the tissue culture dish. The MEFs were then serum starved for 24 hours, extracted into sample buffer, and the status of Pak1 phosphorylation was analyzed by 2-D gel analysis. Under conditions of serum starvation, Pak1 was not activated in control-treated MEFs, as demonstrated by detection of only hypo-phosphorylated forms of Pak1. However, in the ad-Cre treated $Nf2^{flox2}$/- MEFs, which had lost the expression of merlin, a marked activation of Pak1 was observed, as indicated by the appearance of several phosphorylated forms of the kinase. Thus, loss of merlin expression in MEFs promoted Pak1 activation under conditions normally associated with inactivity. These data are consistent with merlin functioning as an inhibitor of Pak1.

Since loss of merlin expression resulted in the appearance of activated forms of Pak1, the Jacks laboratory examined the possibility that overexpression of merlin inhibits Pak1 activation. In the RT4-67 Schwann cells basal levels of merlin are extremely low (29). To assess Pak1 activity in these cells, RT4-67 cells were serum starved for 24 hours, protein was extracted and the activation status of Pak1 was examined by 2-D gel analysis. Expression of merlin was induced by the addition of doxycycline into the growth media 48 hours prior to the harvesting of the cells, in the same manner described above. Indeed, increased expression of merlin significantly reduced the levels of activated forms of Pak1. Thus, the reintroduction of merlin into the RT4 schwannoma cells, which display a high level of basal Pak1 activity, results in inhibition of Pak1 activation.

Over-expression of merlin has been shown by Dr. McClatchey's group to inhibit Rac-induced activation of c-Jun-N-terminal kinase (JNK) and AP-1 transcription, while loss of merlin further resulted in elevated JNK basal and activity of AP-1. Loss of merlin can also induce cytoskeleton changes that are phenotypically consistent with Rac activation, including membrane ruffling and increased cellular motility (30). These new data from the Jacks laboratory, which were published in a recent paper that is appended to this report (31), demonstrate a direct connection between merlin and the Rac-signaling pathways, via the inhibition of Pak. In addition, an established tumor suppressor in the process of Pak1 regulation was identified, possibly linking Pak deregulation to tumorigenesis. Understanding the regulation of merlin by Rac/cdc42 and merlin's impact on these signaling pathways could lead to a more complete understanding for the role of merlin in tumor formation. Once these interactions are fully elucidated, the use of specific inhibitors can be assessed as therapeutic modalities for tumors bearing mutations in *Nf2/NF2*. Such studies are anticipated in the different mouse models of NF2 tumors that have been developed or are under development by members of this consortium.

Biochemical Analysis of Potential Targets for *Nf2* Therapeutics. A better understanding of the molecular effects of merlin deficiency is emerging; the links to Rac signaling and AJ formation provide important potential therapeutic avenues. Drs. McClatchey and Giovannini have begun to examine these pathways in Schwann cells and in schwannomas from the $Nf2^{flox2/flox2}$ mice. Dr. McClatchey's lab has found that basal levels of JNK activity are elevated in primary $Nf2^{-/-}$ Schwann cells as they are in fibroblasts, suggesting that merlin also controls Rac activity in Schwann cells. Given the links between merlin function, cell:cell communication and the actin cytoskeleton her lab will also examine the integrity of AJs in $Nf2^{-/-}$ Schwann cells. It is interesting to note that Schwann cells express AJ components but use them to form specialized *intra*cellular junctions between consecutive wraps as they myelinate (32). Dr. McClatchey is currently investigating whether *Nf2*-deficiency in Schwann cells prevents intracellular AJ formation and myelination (differentiation), leading to persistent proliferation (see below). Her laboratory has already successfully established Schwann cell:neuron cocultures for evaluating the myelinating capability of $Nf2^{-/-}$ Schwann cells in vitro (Figure 5). Notably, firm links between Rac activity and AJ formation have been established, suggesting that they may be coordinately controlled by merlin.

Although the phenomenon of contact-dependent inhibition of proliferation has been linked to AJ formation in other cell types, it is not clear how mitogenic signaling is controlled by AJ formation. Consistent with their observation that $Nf2^{-/-}$ cells do not undergo contact-dependent inhibition of proliferation and continue to proliferate at high density, Dr. McClatchey's lab has found that in contrast to wild-type MEFs, $Nf2^{-/-}$ MEFs do not downregulate key mitogenic signaling proteins, such as MEK, MAPK and cyclin D upon reaching confluence (26). Several recent studies indicate that growth factor receptors themselves localize to AJs and are silenced by their formation, thereby mediating contact-dependent inhibition of proliferation (33). Indeed, Dr. McClatchey has found that the levels of membrane-associated phosphotyrosine and notably active (phosphorylated) EGFR, are downregulated at confluence in wild-type but not $Nf2^{-/-}$ MEFs (manuscript in preparation). Guided by these studies, Dr. McClatchey has examined the levels of growth factor receptor signaling in E12.5 DRG-derived $Nf2^{-/-}$ Schwann cells. Preliminary experiments have revealed elevated levels of certain growth factor receptors in $Nf2^{-/-}$ Schwann cells. Perhaps Schwann cells silence growth factor receptor signaling via the formation of *intra*cellular AJs as they myelinate. Dr. McClatchey's laboratory is currently testing this hypothesis.

Dr. McClatchey has generated a panel of adenoviral vectors expressing wild-type and mutant versions of *Nf2,* including the isolated N- and C-terminal halves as well as full-length versions of *Nf2* containing mutations in the major known phosphorylation site (S518A, S518D), a patient-derived mutation that abrogates the growth inhibiting function and AJ localization (L64P) and a small deletion that confers dominant negative activity upon wild-type *Nf2* (ΔBB). To test the hypothesis that AJ localization is *sufficient* for the growth suppressing activity of merlin, she has also fused full-length cadherin directly to wild-type *Nf2;* when expressed, this fusion exhibits a subcellular localization that is indistinguishable from that of cadherin, thereby restricting merlin localization to AJs. Finally, to test the hypothesis that loss of AJs recapitulates the consequences of *Nf2*-deficiency, Dr. McClatchey has generated an adenovirus expressing a well-characterized dominant negative cadherin (H2Kd-dn-cadherin) (34).

Dr. McClatchey has found that reintroduction of $Nf2^{wt}$ but not $Nf2^{L64P}$ expression restores contact-dependent inhibition of proliferation and AJ formation to $Nf2^{-/-}$ MEFs.
In addition, expression of dominant negative $Nf2^{\Delta BB}$ confers loss of AJs to wild-type MEFs (26).

Finally, expression of the cadherin-Nf2 fusion restores contact-dependent inhibition of proliferation to *Nf2*$^{-/-}$ MEFs in a manner indistinguishable from wild-type Nf2. Preliminary experiments indicate that reintroduction of Nf2^{wt} into *Nf2*$^{-/-}$ Schwann cells also restores contact-dependent inhibition of proliferation and growth factor dependence. Together with complementary studies in other cell types, this panel of viruses will be valuable tools for further delineating the function of merlin in Schwann cell growth control.

Finally, Dr. McClatchey's laboratory has recently cloned the mouse homolog of the *Drosophila* tumor suppressor *expanded. Drosophila* expanded has been shown to be a functional and physical interactor of merlin and therefore a potential target of Nf2 therapeutics. Initial experiments in Dr. McClatchey's lab indicate that merlin and expanded can colocalize in cells and that overexpression of expanded, like merlin causes growth arrest. Ongoing experiments aim to generate antibodies that recognize mouse expanded, to determine the effects of over or under-expression of expanded on the actin cytoskeleton, cell:cell communication and proliferation in mammalian cells and to determine whether merlin and mouse expanded physically interact as their *Drosophila* counterparts do. In addition, the engineering of a targeted mutation of the mouse *expanded* locus is underway.

Introduction of Signaling Molecules into Schwann Cells from P0-tv-a Mice. As described above, tv-a receptor expression renders murine cells susceptible to infection by avian retroviruses engineered to drive the expression of signaling molecules *in vivo.* In addition to the *in vivo* delivery of Cre expression into mature Schwann cells described above, Dr. McClatchey will use this system to introduce activated and dominant-negative alleles of signaling molecules into Schwann cells *in vivo.* The studies described above reveal two key targets of merlin function: Rac signaling and AJ formation. Therefore, avian retroviruses expressing dominant active Rac and dominant negative cadherin will be generated and the effects of their expression on Schwann cell proliferation in vivo will be tested using this system. The construction of RCAS-RacV12 and RCAS-H2Kd-dn-cadherin are currently being generated.

Screening Potential Therapeutic Compounds in *Nf2*$^{-/-}$ Cells. The collaborative studies of Drs. McClatchey, Giovannini, and Jacks suggest several possible therapeutic strategies for *Nf2*-associated tumors, which can be investigated in cell-based assays. The first involves inhibiting Rac signaling, either directly through the use of geranyl-geranyl-transferase (GGTase) inhibitors (GGTIs) that interfere with the processing of Rac (35, 36) or indirectly via inhibitors of Ras, PI3K or MAPK signaling which are instrumental in signaling to Rac. A second potential approach involves modulating PKA output. Several cell-permeable compounds that specifically stimulate or inhibit the PKA pathway have been developed, including forskolin and IBMX (activators), as well as H-89 and 8-bromo-cAMP (inhibitors). Given data indicating that the ERM proteins and merlin (Gronholm et al, 2003) can control PKA signaling, the link between PKA and merlin phosphorylation, and the requirement for PKA signaling in Schwann cell proliferation, an exploration of the effects of these compounds on *Nf2*$^{-/-}$ cell growth and viability is warranted.

In pilot experiments that built upon the evaluation of *Nf2*$^{-/-}$ cells, Dr. McClatchey established a cell-based assay for examining the ability of the inhibitors described above to selectively block the proliferation of *Nf2*-deficient cells. Taking advantage of the fact that *Nf2*$^{-/-}$ but not wild-type MEFs continue to grow in the absence of added growth factors, her lab tested the ability of each of the aforementioned signaling inhibitors to block the growth factor

independent proliferation of *Nf2*$^{-/-}$ MEFs. They found that all of the inhibitors tested blocked the persistent proliferation of *Nf2*$^{-/-}$ cells under these conditions, suggesting that no single mitogenic pathway was responsible for the growth factor independence of *Nf2*$^{-/-}$ MEFs (Figure 5). These results also suggest that *Nf2* loss affects signaling upstream of these pathways and support the idea that merlin may control the activity of growth factor receptors themselves from the plasma membrane as described above. Dr. McClatchey is now devising alternative cell-based assays that will provide a more specific readout for *Nf2*-deficiency. For example, it may be possible to devise an assay that measures the restoration of AJs in *Nf2*-deficient cells.

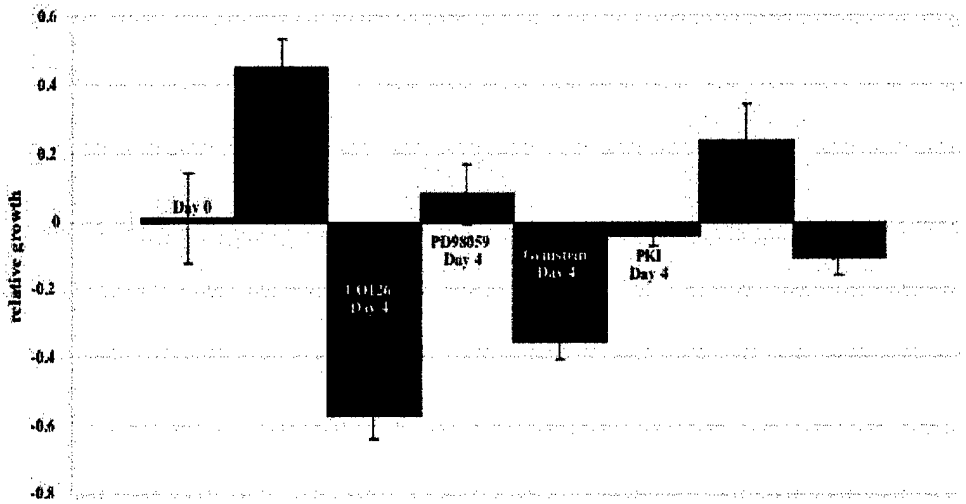


Fig. 5 Effects of signaling inhibitors on the proliferation of *Nf2*$^{-/-}$ MEFs after 4 days of serum starvation. All compounds tested either blocked proliferation or induced apoptosis of serum-starved *Nf2*$^{-/-}$ MEFs. DMSO, vehicle control; U0126, MEK inhibitor; PD98059, MAPK inhibitor; Genestein, tyrosine kinase receptor inhibitor; PKI, PKA activator; GGTI, geranyl-geranyl transferase inhibitor.

Aim 3. Preclinical Studies of Experimental Therapeutics in Mouse Models

Research Plan

Preclinical Evaluation of a MEK Inhibitor in the JMML Model. MEK is a dual specificity kinase that catalyzes the phosphorylation of p44^{MAPK} (ERK1) and of p42^{MAPK} (ERK2). In myeloid cells, MEK is directly activated by Raf and by cross-cascade signaling from the PI3K pathway (data not shown). PD184352 was identified in a screen for small molecule inhibitors of MEK (17). Biochemical studies infer an allosteric mechanism of action. PD184352 is a potent inhibitor of MAPK activation in cancer cell lines, and it induced regression of explanted tumors in nude mice that correlated with *in vivo* effects on MAPK phosphorylation (17). PD184352 is undergoing phase 1 testing in refractory malignancies. Dr. Shannon obtained PD184352 from Pfizer, Inc., and is currently studying this agent in the *Mx1-Cre, Nf1*$^{flox/flox}$ model of JMML. Dr. Shannon has shown that 0.01 μM to 10 μM of PD184352 abrogates CFU-GM colony formation

in response to GM-CSF from normal murine bone marrow as well as from wild-type and *Nf1*$^{-/-}$ fetal livers (data not shown). A 4 week toxicity study in wild-type mice in which treated animals received twice daily doses of 100 mg/kg of PD184352 showed that the drug was tolerated at this dose and blocked ERK activation by GM-CSF for ~4 hours. Thus, in contrast to FTI, PD184352 markedly inhibits a relevant biochemical target in primary *Nf1* mutant cells at tolerable doses. A controlled preclinical study was then performed in which this schedule was administered to *Mx1-Cre Nf1*$^{flox/flox}$ mice with MPD. Although PD184352 was well tolerated and we could demonstrate ERK kinase inhibition 2 and 4 hours after treatment, there was no beneficial effect on the MPD. We believe that better target inhibition will be required for efficacy; however, PD184352 is difficult to administer more then twice daily and cannot be given through an implantable device. We are preparing these ifindings for publication and hope to obtain and screen better compounds in the tractable *Mx1-Cre Nf1*$^{flox/flox}$ model.

Aim 4. To sponsor specialized working group meetings and to support preclinical testing in mouse models of NF1 and NF2.

Workshop on Pathologic Classification of Tumors in NF Mouse Models. Drs. Small and Giovannini organized a meeting of pathologists and basic scientists on February 8-10, 2003, in Boston, Massachusetts, to review all available genetically engineered murine (GEM) models of NF1 and NF2, and to draft a pathological classification of murine peripheral nerve sheath tumors based on these models. Dr. Anat Stemmer-Rachamimov (Harvard University), who has extensive expertise in NF-associated tumors, led a panel of 10 pathologists with different areas of emphasis that reviewed lesions from most published and unpublished NF mouse models and compared their histological features with the corresponding human tumors. The aim of the workshop was to review all available peripheral nerve sheath tumors (PNSTs) from the neurofibromatosis type 1 and type 2 murine models generated to date, and to develop a pathological classification to characterize these lesions in a uniform and consistent manner. The meeting and pathological classification followed a format successfully adopted by similar pathology workshops for murine models of other organ systems. Pathologists with different areas of expertise reviewed 56 lesions from 8 laboratories representing most published and unpublished NF mouse models and compared their histological features with the corresponding human tumors. In formulating the GEM classification of peripheral nerve sheath tumors, the panel referred to criteria and terminology devised by the World Health Organization for classifying human nervous system tumors (37). However, although the WHO classification served as a useful point of reference, it was not adopted for the classification of the GEM lesions because of some important differences between the human and murine tumors. The WHO classification of human peripheral nerve sheath tumors (PNSTs) is based on both histological and clinical characteristics (and describes clinico-pathological entities), whereas the GEM classification must necessarily be based on histological features alone, as clinical features of murine PNSTs are not yet known. Furthermore, although many of the histological features displayed by GEM PNSTs were similar to those observed in human tumors, some GEM lesions presented unique and distinct morphology, and did not fit into the human classification by histological criteria.

In addition, because the human tumor nomenclature refers to histological features as well as predicted clinical behavior of tumors, some of these terms were deemed inappropriate for description of GEM tumors, in which biological behavior is still unknown. The panel therefore proposed a novel classification for GEM nerve sheath tumors based purely on morphological features. Although the term "grade" is used in the GEM classification, it is applied as a

histological descriptor only and has no predictable bearing on the biological behavior of the tumor in the animals. In other words, as opposed to the WHO classification where grade is an indicator of biological behavior, grading in the GEM classification refers merely to the presence or absence of specific histological features such as high cellularity, necrosis, nuclear pleomorphism and brisk mitotic activity.

This classification represents a valuable tool for standardization and validation of tumors arising in NF mouse models, as it facilitates direct comparisons between models and enables effective design and interpretation of preclinical trials that are directly relevant to human NF disease. Finally, the panel also recommended general guidelines for the work-up and morphological evaluation of peripheral nerve sheath lesions in GEM models. A manuscript is in preparation, which described the guidelines developed through this highly interactive and collaborative effort.

Workshop on Preclinical Therapeutics in NF Mouse Models. Dr. Small and the NNFF staff will organize a second workshop during year 2 that will be held in year 2 or 3. This meeting will explore how mouse models of NF-associated might be used to evaluate therapeutics. Dr. Shannon, who is playing a leading role in this area within the MMHCC, will co-chair this meeting with Dr. Small and with an expert in human clinical trial design.

KEY RESEARCH ACCOMPLISHMENTS

(a) The investigators continue to extensively share expertise and reagents to pursue common research goals.

(b) The NF Modeling Group was admitted to the NCI MMHCC and is contributing to this important national effort.

(c) We have developed accurate models of most NF1 and NF2-assocated tumors, have characterized these lesions, and are pursuing the goals of developing additional models and of enhancing existing mutant strains.

(d) Studies in *Krox20 Nf1*$^{flox/flox}$ mice demonstrated that a heterozygous mutant environment plays a major role in neurofibroma formation.

(e) Pak-1 was shown to be is regulated by merlin, thus establishing this signaling kinase as an attractive therapeutic target.

(f) The growth of *Nf2*-deficient fibroblasts was shown to be independent of exogenous growth factors, and preliminary data indicate that the *Nf2* mutant Schwann cells proliferate in the absence of GGF.

(g) A new method was developed for isolating mitotically active Schwann cells.

(h) A comprehensive *in vivo* analysis of *Rce1*-deficient hematopoietic cells demonstrated that inactivation of this Ras processing enzyme is not associated with impaired growth.

(i) *Mx1-Cre Nf1*$^{flox/flox}$ mice provide a tractable model of JMML that is being harnessed to test an inhibitor of MEK *in vivo* with correlative biochemical (pharmacodynamic) monitoring.

(m) Strains of mutant mice have been shared widely with the NF research community (see list below in Reportable Outcomes). Through these collaborative experiments, the scientific value of this Consortium has extended well beyond the studies being pursued in the participant's laboratories.

REPORTABLE OUTCOMES

(a) Previous Research Articles and Reviews (2000 – 2002)

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Submitted Research Articles

Le DT, Kong N, Zhu Y, Aiyigari A, Braun BS, Wang E, Kogan SC, Le Beau MM, Parada L, Shannon KM. Somatic inactivation of *Nf1* in hematopoietic cells results in a progressive myeloproliferative disorder. *Blood* (in revision)

Romero' M.I., Zhu' Y., Lush, M.E., and Parada, L.F. 2003 Neuron-specific deletion of NF1 enhances functional recovery after spinal cord Sensory denervation. Submitted

Zhu, Y., Harada, T., Guignard, F., Harada, C., Burns, D. K., Bajenaru, M.L, Gutmann, D.H., Messing, A., and Parada, L.F. 2003 Ablation of NF1 in CNS causes transient neural progenitor hyperplasia and is sufficient to induce optic gliomas. Submitted.

(b) Model Development and Distribution to the Research Community

As described in the Body of this application, studies conducted to date have established a number of novel models of NF1 and NF2-associated tumors and have generated several new strains of mice. *Nf1* and *Nf2* mutant mice have been deposited in the MMHCC Repository where they are

readily available to the research community. In addition, the participants in this Consortium have provided strains directly to the investigators listed below.

Karlene Reilly (National Cancer Institute)
Jeffrey DeClue (National Cancer Institute)
Jonathan Epstein (University of Pennsylvania)
D. Wade Clapp (Indiana University)
David Guttmann (Washington University)
David Largaespada (University of Minnesota)
Jeffrey Lawrence (UCSF)
Alcino Silva (UCLA)
Gerard Karsenty (Baylor)
Shaojun Tang (UC Irvine)
Shalom Avraham (Beth Israel)
James Bieker (Mount Sinai, New York)
Abhijit Guha (Labatt Brain Tumor Research Center, Tornoto)
Andreas Kurtz, (Harvard)
Jim Gussella (Harvard)
Dan Haber (Harvard)
Antonio Chiocca (Harvard)
Isidro Sanchez-Garcia (IBMCC)
Victor Tybulewicz (National Institute for Medical Research, London)
Lindsay Hinck (UC Santa Cruz)
Keqiang Ye (Emory University School of Medicine)
Lynda Chin (Dana Farber Cancer Institute)
Joseph Testa (Fox Chase Cancer Center)
Nancy Ratner (U. of Cincinnati)
Stefan Mundlos (U. of Berlin)
Juha Peltonen (U. of Helsinki, Finland)

(c) Employment and Research Opportunities

This award has provided salary support for technical personnel in each of participating labs.

CONCLUSIONS

During the third year of its existence, this consortium made progress in accomplishing its primary goal of generating and characterizing mouse models of NF1 and NF2-associated tumors for biologic and preclinical therapeutic trials. A number of novel strains have been developed and reported, innovative strategies were deployed to make optimal use of these resources, and our recent research has provided a number of novel insights regarding potential therapeutic targets in NF disease. The investigators have collaborated closely and have shared expertise and reagents extensively. Dr. Giovanini organized a highly successful meeting that focused on Pathologic Classification of Mouse Models of NF-Associated Tumors. This NF Consortium is a full participant in the Mouse Models of Human Cancer Consortium of the NCI.

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Brief report

Hematologic effects of inactivating the Ras processing enzyme *Rce1*

Abigail L. Aiyagari, Brigit R. Taylor, Vikas Aurora, Stephen G. Young, and Kevin M. Shannon

Posttranslational processing of Ras proteins has attracted considerable interest as a potential target for anticancer drug discovery. *Rce1* encodes an endoprotease that facilitates membrane targeting of Ras and other prenylated proteins by releasing the carboxyl-terminal 3 amino acids (ie, the -AAX of the CAAX motif). Homozygous *Rce1* mutant embryos ($Rce1^{-/-}$) die late in gestation. To characterize the role of *Rce1* in hematopoiesis, we performed adoptive transfers and investigated cells from the recipients. $Rce1^{-/-}$ fetal liver cells rescued lethally irradiated recipients and manifested normal long-term repopulating potential in competitive repopulation assays. The recipients of $Rce1^{-/-}$ cells developed modest elevations in mature myeloid cells (neutrophils + monocytes), but remained well. Bone marrow cells from mice that received transplants of $Rce1^{-/-}$ activated extracellular signal-related kinase (ERK) normally in response to granulocyte-macrophage colony-stimulating factor. These data suggest that pharmacologic inhibitors of Rce1 will have minimal effects on normal hematopoietic cells. (Blood. 2003;101:2250-2252)

Introduction

Ras proteins regulate cell fates by cycling between an active guanosine 5c-triphosphate (GTP)-bound state and an inactive guanosine 5c-diphosphate (GDP)-bound state in response to extracellular stimuli, including activation of hematopoietic growth factor receptors.[1-3] *RAS* genes are mutated frequently in myeloid malignancies and in other human cancers (reviewed in Bos[4] and Rodenhuis[5]). In addition to *RAS* point mutations, inactivation of the *NF1* tumor-suppressor, expression of the Bcr-Abl fusion, and mutations of the *FLT3* receptor are thought to contribute to leukemogenesis, at least in part, by deregulating Ras signaling.[6-8] While these data establish hyperactive Ras as a therapeutic target, disrupting Ras function may have adverse effects on normal tissues.

Ras proteins undergo posttranslational modification at a common C-terminal CAAX sequence (reviewed in Gibbs and Oliff,[9] Sebti and Hamilton,[10] and Le and Shannon[11]). Processing is initiated by farnesyltransferase (Ftase), which attaches a farnesyl lipid to the thiol group of the cysteine (the "C" of the CAAX motif). Prenylation targets Ras to membranes, and is required for the biologic activity of normal and oncogenic Ras. Ftase inhibitors have shown promise as anticancer agents.[10-13] However, K-Ras and N-Ras are substrates for geranylgeranyltransferase 1 (GGTase 1) and are processed by this alternative pathway when Ftase is inhibited. Indeed, extensive data now support the view that non-Ras CAAX proteins are critical in vivo targets of the Ftase inhibitors (reviewed in Sebti and Hamilton,[10] Le and Shannon,[11] and Omer et al[14]). After prenylation, the carboyxl terminal 3 amino acids are released by Rce1, an integral membrane endoprotease of the endoplasmic reticulum. The final step in Ras processing involves methylation of the prenylcysteine by isoprenylcysteine carboxyl methyltransferase.

The murine *Rce1* gene was disrupted to elucidate its role in development and tumorigenesis.[15] Homozygous mutant embryos ($Rce1^{-/-}$) demonstrated late embryonic lethality with normal organogenesis.[15] To define the importance of *Rce1* in hematopoiesis, we have performed adoptive transfer, biochemical, and competitive repopulation experiments.

Study design

Mice

Rce1 knock-out mice have been described.[15] C57Bl/6 mice and congenic B6.SJL-PtrcaPep3b/BoyJ (B6/BoyJ) mice (CD45.1$^+$) were purchased from Jackson Laboratory (Bar Harbor, ME). B6/BoyJ mice express a variant allele of the CD45 cell surface protein (CD45.1). The experimental procedures were approved by the UCSF Committee on Animal Research.

Adoptive transfer and competitive repopulation

Heterozygous *Rce1* mutant ($Rce1^{+/-}$) mice were mated to produce wild-type, $Rce1^{+/-}$, and $Rce1^{-/-}$ embryos. Fetal liver cells were isolated and injected into irradiated 8- to 12-week-old recipients as described.[15,16] The competitive repopulation methodology was based on published protocols.[17] Briefly, recipients received a mixture of C57Bl/6 CD45.2 $Rce1^{-/-}$ or wild-type fetal liver tester cells (1×10^5 to 2×10^6, per mouse) with a standard number of congenic BoyJ CD45.1 competitors (5×10^5 per mouse). Mice injected with only competitor cells consistently showed less than 5% autologous recovery.

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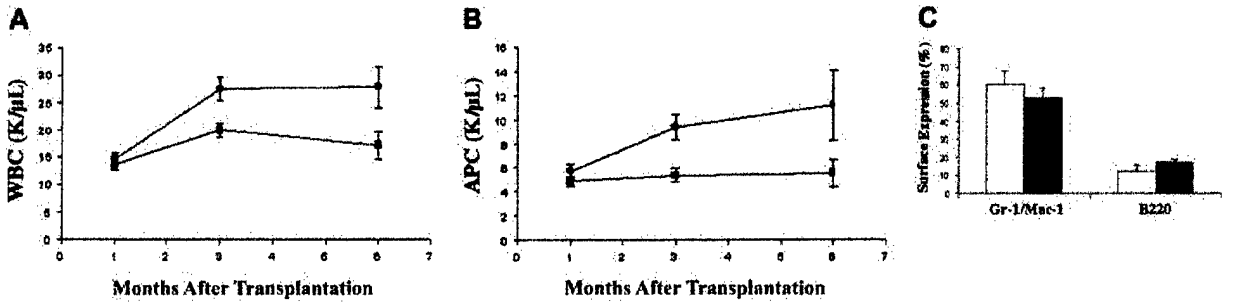

Figure 1. Hematologic parameters in recipients of *Rce1*$^{-/-}$, *Rce1*$^{+/-}$, and wild-type fetal liver cells. Recipients of *Rce1*$^{-/-}$ cells (n = 32) are shown as ● in panels A and B and as □ in panel C. We observed no differences in recipients of *Rce1*$^{+/-}$ and wild-type fetal liver cells, so the data from these mice were pooled (n = 40) and are shown as ■ in panels A and B and as ■ in panel C. (A) White blood cell counts (WBCs). (B) Absolute phagocyte counts (APCs; monocytes + neutrophils). (C) Relative numbers of bone marrow cells expressing the myeloid markers Gr-1 and Mac-1 and the B-lymphocyte marker B220 6 months after adoptive transfer.

Monitoring

Complete blood cell counts were measured in a Hemavet instrument (CDC Technologies, Oxford, CT) and differential counts were confirmed by examining blood smears stained with Wright-Giemsa. For chimerism studies, blood and bone marrow cells were stained with allele-specific antibodies to CD45.1 and CD45.2 (Pharmingen, San Diego, CA) followed by fluorescence activated cell staining (FACS) analysis. In some experiments, cells were also labeled with antibodies that recognize CD3 (T lymphocytes), B220 (B lymphocytes), Gr-1 (granulocytes), and Mac-1 (myelomonocytic cells) (Pharmingen). Using a Becton Dickinson FACScan, 10 000 events were collected and the data were analyzed using FlowJo (Tree Star, San Carlos, CA) and Cell Quest software (Becton Dickinson, Mountain View, CA).

Colony assays

Colony-forming units-granulocyte macrophage (CFU-GMs) were assayed in methylcellulose medium (Stem Cell Technologies, Vancouver, BC, Canada) containing murine granulocyte-macrophage colony-stimulating factor (GM-CSF) concentrations. Colony growth was scored on day 8.

Kinase assays

Lysates were prepared from bone marrow as described.[7,18] Protein concentrations were equalized using the Bradford Colorimetric Assay (Pierce Chemical, Rockford, IL) and equal loading was confirmed by Western blotting. Extracellular signal-related kinase (ERK) was immunoprecipitated with a specific antiserum (Cell Signaling, Beverly, MA; catalog no. 9101) and kinase immune complex assays were performed as described[18] using an Elk1 fusion protein (New England Biolabs, Beverly, MA) as a phosphorylation substrate.

Results and discussion

Fetal liver cells of all *Rce1* genotypes efficiently rescued hematopoiesis in irradiated recipients. Because leukocyte counts in mice injected with wild-type or *Rce*$^{+/-}$ cells were similar, these groups were combined for analysis. Mice engrafted with *Rce1*$^{-/-}$ fetal liver cells developed leukocytosis by 3 months that persisted until they were killed at 6 months (Figure 1A). This was due to increased numbers of mature myeloid cells in the *Rce1*$^{-/-}$ recipients (Figure 1B). These mice remained well, and demonstrated normal spleen sizes and splenic architecture at killing (data not shown). However, FACS analysis of marrow and spleen from recipients of *Rce1*$^{-/-}$ fetal liver cells demonstrated an increase in the percentage of myeloid cells (Gr-1 and/or Mac-1–positive) and a commensurate reduction in B lymphocytes (B220-positive) (Figure 1C). Because GM-CSF promotes the growth of myelomonocytic cells, we assayed CFU-GM growth in methylcellulose over a range of GM-CSF concentrations. Although there was some variability between individual experiments, colony growth was similar for wild-type, *Rce1*$^{+/-}$, and *Rce1*$^{-/-}$ cells. *Rce1*$^{-/-}$ CFU-GM colonies grown in saturating concentrations of GM-CSF demonstrated less spreading than wild-type colonies.[15]

ERK activities were measured in bone marrow collected 3 to 6 months after adoptive transfer. In multiple experiments, wild-type and *Rce1*$^{-/-}$ cells demonstrated equivalent basal and GM-CSF–stimulated ERK activities (Figure 2A).

Wild-type and *Rce1*$^{-/-}$ fetal liver tester cells were injected into irradiated hosts with the same reference population of BoyJ competitor cells to directly compare their repopulating potentials. Cells of both genotypes demonstrated equivalent repopulating potentials over a dose range that produced 10% to 70% donor cell chimerism (Figure 2B). These data provide strong evidence that inactivation of *Rce1* does not impair the proliferative capacity of hematopoietic cells.

As the only known CAAX protease in mammalian cells, Rce1 represents an attractive target for cancer drug discovery. Genetic ablation eliminates Ras endoproteolytic activity, which results in mislocalization of approximately 50% of Ras away

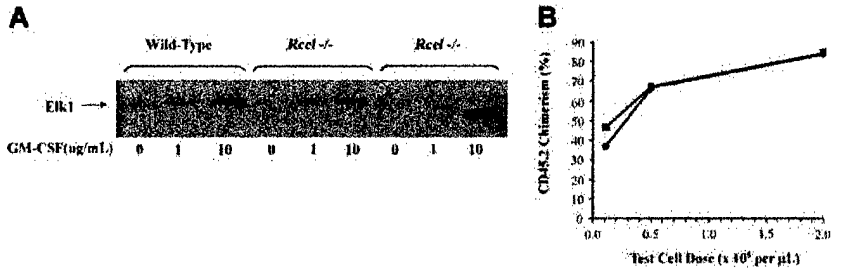

Figure 2. ERK activation and repopulating potential of *Rce1*$^{-/-}$ cells. (A) Bone marrow was collected 3 to 6 months after adoptive transfer, incubated under low (0.1%) serum conditions for 4 hours, and then stimulated with recombinant murine GM-CSF. The cells were lysed after 10 minutes, immunoprecipitated with an antibody against ERK, and phosphorylation of an Elk1 substrate was measured. (B) Percentages of peripheral blood leukocytes derived from *Rce1*$^{-/-}$ (■) and wild-type (●) 4 months after adoptive transfer with a standard population of CD45.1 competitor cells. Data are pooled from independent experiments and represent 3 to 6 recipients at each point.

from the plasma membrane.[15] Importantly, *Rce1*-deficient cells are unable to process either farnesylated or geranylgernaylated substrates.[15] Our data suggest that the amount of Ras that is correctly targeted to the plasma membrane in *Rce1*$^{-/-}$ cells is sufficient for normal signal output from activated cytokine receptors, which is consistent with the low density of many receptors on hematopoietic precursors. Alternatively, hematopoietic cells may express another protease that can compensate for the loss of Rce1. It is unclear why recipients of *Rce1*$^{-/-}$ transplants demonstrated modest proliferation of mature myeloid elements; however, many cellular proteins in addition to Ras are substrates for Rce1. This modest myeloid proliferation raises the possibility that inhibiting Rce1 might paradoxically stimulate the growth of myeloid malignancies.

At first glance, these results argue that Rce1 represents a poor therapeutic target in leukemia, particularly when genetic lesions such as the *BCR-ABL* translocation, loss of *NF1*, or mutations of *FLT3* lead to aberrant activation of normal Ras proteins. However, lack of toxicity to normal hematopoietic cells also represents a distinct advantage and it is noteworthy that STI-571 (Gleevec) is remarkably selective for cells that express Bcr-Abl despite fully inhibiting c-Abl and c-kit.[19] Similarly, it is possible that blocking Rce1 will differentially affect cells that express normal versus mutant Ras proteins. Studies in tissue culture and in transgenic mice have shown that transformed cells select for higher levels of oncogenic Ras[20,21]; this may render them sensitive to a modest reduction in Ras signaling. Furthermore, because oncogenic Ras accumulates in the GTP-bound conformation, mislocalizing it away from the plasma membrane might sequester effectors such as Raf. Interestingly, somatic deletion of *Rce1* was recently associated with a reduction in *Hras*- and *Kras*-induced transformation in soft agar and with a competitive disadvantage of skin carcinoma cells expressing oncogenic *Hras*.[22] We are exploiting this conditional mutant allele to investigate the effects of inactivating Rce1 in genetically engineered mouse models of myeloid malignancies associated with hyperactive Ras.

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APPENDIX 2

Merlin, the Product of the Nf2 Tumor Suppressor Gene, Is an Inhibitor of the p21-Activated Kinase, Pak1

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Summary

The Nf2 tumor suppressor gene codes for merlin, a protein whose function has been elusive. We describe a novel interaction between merlin and p21-activated kinase 1 (Pak1), which is dynamic and facilitated upon increased cellular confluence. Merlin inhibits the activation of Pak1, as the loss of merlin expression results in the inappropriate activation of Pak1 under conditions associated with low basal activity. Conversely, the overexpression of merlin in cells that display a high basal activity of Pak1 resulted in the inhibition of Pak1 activation. This inhibitory function of merlin is mediated through its binding to the Pak1 PBD and by inhibiting Pak1 recruitment to focal adhesions. This link provides a possible mechanism for the effect of loss of merlin expression in tumorigenesis.

Introduction

Neurofibromatosis type 2 is an inherited disorder, characterized by development of Schwann cell tumors of the eighth cranial nerve. Mutations and loss of heterozygosity of the *NF2* locus have been detected at high frequency in various tumors of the nervous system, including schwannomas, meningiomas, and ependymomas (Gusella et al., 1999). Mice heterozygous for an *Nf2* mutation are predisposed to a wide variety of malignant tumors (McClatchey et al., 1998). Inactivation of *Nf2* specifically in Schwann cells leads to development of schwannomas and Schwann cell hyperplasia in mice (Giovannini et al., 2000). The *NF2* gene codes for a 595 amino acid protein, termed merlin, which is highly related to the ERM proteins ezrin, radixin, and moesin.

Recent work has shown that merlin protein levels and phosphorylation are affected by growth conditions such as cell confluence, loss of adhesion, or serum deprivation. One site of phosphorylation of merlin is serine 518, and phosphorylation at this site can be induced by active forms of Rac and cdc42 but not Rho (Shaw et al., 2001). Rac/cdc42-induced phosphorylation at merlin serine 518 is mediated by p21-activated kinase (Pak) (Kissil et al., 2002; Xiao et al., 2002). Such phosphorylation can disrupt merlin intramolecular interactions and its association with the actin cytoskeleton and induces a shift in the subcellular localization of merlin in LLC-PK1 cells (Kissil et al., 2002; Shaw et al., 2001).

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The p21-activated kinases (Pak1 through 3) are immediate downstream effectors of Rac/cdc42. They comprise a subgroup of serine/theronine kinases, termed the "group I" Paks, belonging to a larger protein family, which also contains the "group II" kinases (Pak4, 5, and 6). The group I Paks, which have been studied in more detail, have been shown to mediate signals to cytoskeletal reorganization and transcriptional activation (reviewed by Bagrodia and Cerione, 1999; Jaffer and Chernoff, 2002). The Paks are regulated by diverse mechanisms. Based on three-dimensional structure analysis, it has been suggested that inactive Pak is in a conformation in which the autoinhibitory domain interacts with the kinase domain. The binding of active Rac/cdc42 to Pak alleviates this inhibition and enables Pak activation. Once the inhibition is relieved, Pak undergoes autophosphorylation, and this prevents a conformational switch back into an inactive state (Li et al., 2001). Several additional mechanisms and molecules have been shown to regulate Pak activation, including phospholipids and proteolysis. In addition, membrane localization via NCK, localization to focal adhesions via p95/PKL-Cool/Pix, and signals converging from both growth factor receptors and integrins can all effect Pak activation (Brown et al., 2002; del Pozo et al., 2000; Turner et al., 1999).

Recently, merlin has been implicated as a negative regulator of Rac signaling. The overexpression of merlin inhibited Rac-induced activation of c-Jun N-terminal kinase (JNK) and activation of the AP-1 transcriptional activator. Conversely, in Nf2-deficient fibroblasts, basal JNK activity was found to be elevated, as was the activity of AP-1 (Shaw et al., 2001). Thus, it would seem that merlin both is regulated by the Rac/cdc42 signaling pathway and can serve as an inhibitor of this pathway. Here we describe the interaction of merlin with Pak1, a critical mediator of Rac/cdc42 signaling, and the effect of this interaction on the activity of the kinase.

Results and Discussion

Direct Interaction between Merlin and Pak1

Based on the observations that merlin inhibits Rac signaling at some level, we assessed the possibility of a stable interaction between merlin and Pak. NIH3T3 cells were cotransfected with expression vectors for merlin and Pak1, and association of the proteins was assessed by coimmunoprecipitations. As shown in Figure 1A, immunoprecipitation of merlin led to coimmunoprecipitation of Pak1. Likewise, immunoprecipitates of Pak1 contained merlin (Figure 1A). To assess the interaction in an additional cell type, a rat schwannoma cell line (RT4-DP6) was examined. These cells express relatively low levels of endogenous merlin and detectable endogenous levels of Pak1. Neither Pak2 nor Pak3 was detected in these cells by Western blot analysis (data not shown). As in the case of NIH3T3 cells, association of the proteins was assessed by coimmunoprecipitations. As shown in Figure 1A, the immunoprecipitation of merlin also precipitated endogenous Pak1. In the reciprocal experi-

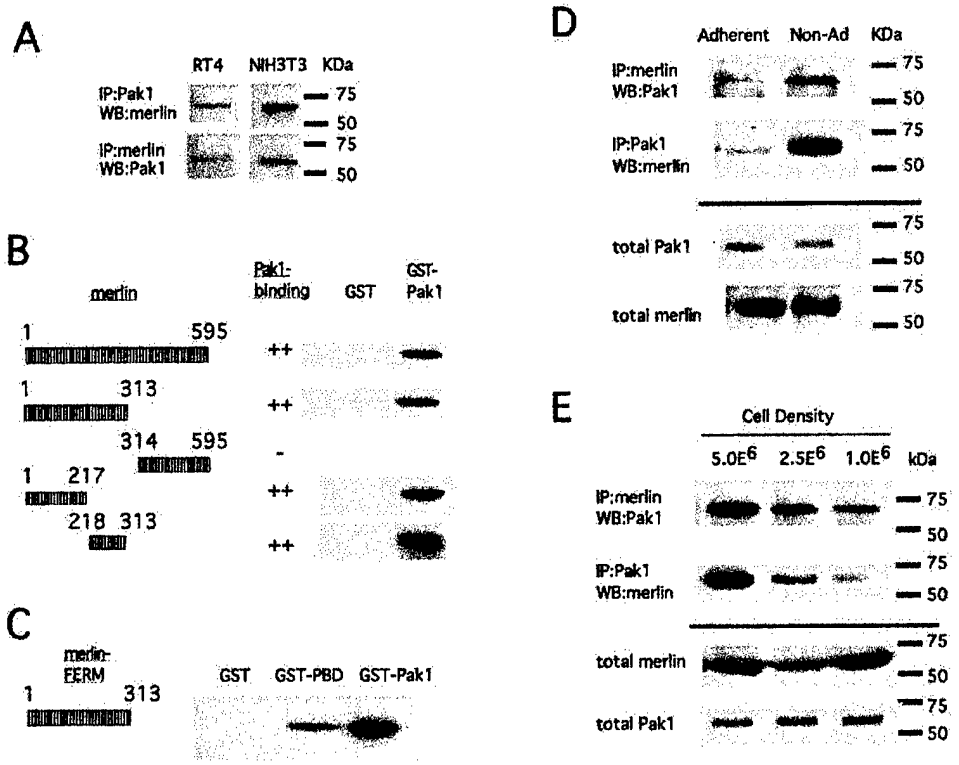

Figure 1. Merlin and Pak1 Interact In Vitro and In Vivo

Western blot analysis of merlin and Pak1 immunoprecipitates from NIH3T3 cells transfected with expression vectors for merlin and Pak1, and from RT4 Schwann cells expressing endogenous levels of merlin and Pak1 (A). In vitro interactions of GST only or GST-Pak1 proteins with S^{35}-labeled full-length merlin (1–595), FERM domain (1–313), C-terminal half (314–595), FERM lobes F1-F2 (1–217), and lobe F3 (218–313) (B). In vitro interactions of the merlin FERM domain with GST, GST-Pak1 (1–545), and GST-PBD (70–143) (C). Western blot analysis of merlin and Pak1 immunoprecipitates and total expression levels of protein from RT4-67 grown under adherent or nonadherent growth conditions for 4 hr prior to harvesting (D) or grown at increasing cellular densities as indicated (E).

ment, the immunoprecipitation of endogenous Pak1 also resulted in the coimmunoprecipitation of endogenous or exogenous merlin (Figure 1A).

To determine if merlin and Pak can interact directly, the association of the two proteins was assessed in vitro. Full-length Pak1 was produced in bacteria as a GST-fusion protein and purified on glutathione- agarose beads. Merlin was produced by in vitro transcription/translation. The S^{35}-labeled merlin protein was incubated with either GST-Pak1 or GST bound to the agarose beads. The interaction of the proteins was assessed by separation of the proteins by SDS-PAGE and autoradiography. As shown in Figure 1B, GST-Pak1 bound to merlin, whereas GST alone did not. Thus, the interaction between Pak and merlin is likely to be direct.

To further delineate the regions of Pak1 that mediate interaction with merlin, we assessed the binding of different merlin domains to full length Pak1 in vitro. The N-terminal FERM domain (1–313) and the C-terminal tail of merlin (314–595) were transcribed and translated in vitro and tested for their ability to interact with GST-Pak1. While the N-terminal FERM domain interacted efficiently with GST-Pak1, the C-terminal fragment failed to interact (Figure 1B). This was not due to different levels of expression, as all merlin fragments were produced at similar levels (data not shown). Trying to further narrow down the interacting domains in the merlin FERM, we tested the ability of either the F1-F2 domain (1–217) or the F3 domain (218–313) (Pearson et al., 2000) to bind to Pak1. Both domains interacted well with the kinase, indicating there are multiple binding sites in the FERM domain involved in the interaction of merlin with Pak1 (Figure 1B). The interaction of both the F1-F2 and F3 fragments with GST-Pak1 appeared to be stronger than that of the entire FERM domain (F1-F3). This could be due to intramolecular associations within the intact FERM domain (Gutmann et al., 1999) that partially mask the Pak1 interaction sites. We next tested the possibility that the FERM domain could bind to the N-terminal regulatory domain of Pak1 (70–143), which contains the cdc42/Rac binding domain (PBD). The FERM domain interacted with the PBD, although the interaction was weaker than the interaction with full-length Pak1.

We attempted to identify additional merlin binding sites on Pak1 by generating additional truncation mutants; however, we were unable to obtain these mutants due to the high toxicity of the Pak1 kinase domain in bacteria (J. Chernoff and E. Manser, personal communication). Thus, it remains possible that additional interaction domains exist between merlin and Pak1. However, the identification of an interaction between the FERM domain with the PBD is of functional significance, as it implicates merlin in a regulatory role for Pak1 (see below).

The Interaction of Merlin and Pak Is Dynamic

To assess whether the interaction between Pak and merlin is dynamic, we examined the effect of cell adhesion and confluence on the interaction. Exogenous expression of merlin was employed in these experiments to circumvent the fact that merlin expression is regulated by different cellular growth conditions (Shaw et al., 1998). Toward this aim, the RT4-67 cell line was employed. The RT4-67 cells were constructed from the RT4-DP6 rat schwannoma cells and harbor a tetracycline-inducible allele of NF2 (Morrison et al., 2001). RT4-67 cells were grown in the presence of doxycycline and placed into suspension by plating them on poly-HEME coated dishes, which prevents the cells from adhering to the plastic (Folkman and Moscona, 1978). Four hours after being placed into suspension, the cells were harvested, and either merlin or Pak1 was immunoprecipitated. The precipitates were resolved by SDS-PAGE and Western blotting and compared to precipitates from adherent RT4-67 cells. While merlin and Pak1 could be coimmunoprecipitated under adherent growth conditions, the interaction between the two proteins was greatly enhanced when adhesion was lost (Figure 1D).

To test the effect of cell confluence on the merlin-Pak1 interaction, protein extracts were prepared from RT4-67 cells grown at high or low confluence. The cells were plated at increasing densities in the presence of doxycycline and were harvested 24 hr after plating. Merlin or Pak1 was immunoprecipitated from the cellular extracts and resolved by SDS-PAGE. Both merlin and Pak1 can reciprocally coimmunoprecipitate under conditions of either low or high confluence. However, the interaction between merlin and Pak1 was enhanced when cells were grown to a higher density (Figure 1E). Importantly, the observed differences in the precipitated levels of merlin or Pak1 are not due to differences in the expression levels of these proteins. The levels merlin and Pak1 were not altered in the RT4-67 Schwann cells, whether the cells were adherent or nonadherent or grown at high or low cellular densities.

These experiments indicate that the interaction of merlin and Pak1 is dynamic and influenced by cellular adhesion and cell density. The interaction of merlin and Pak was enhanced under conditions demonstrated to be inhibitory to Pak activation in NIH3T3 cells (del Pozo et al., 2000) and in the RT4-67 Rat schwannoma cells (J.L.K. and T.J., unpublished data). The regulation of the Paks is complex and involves many different factors (discussed below). However, the localization of Pak to specific regions at the plasma membrane might be an important determinant of its activation. Earlier work has shown that the SH2/SH3 domain protein NCK is required for the recruitment of Pak to the cell membrane (Lu et al., 1997; Sells et al., 1997). Interestingly, NCK interaction with Pak is enhanced upon adhesion and lost when cells are suspended (Howe, 2001), which is opposite to the pattern of interaction of merlin and Pak shown here.

Merlin Inhibits the Pak1-Rac and Pak1-Paxillin Interactions

As merlin bound the Pak1 PBD domain, we tested the possibility that merlin can inhibit the interaction between Rac and Pak1. RT4-67 cells were grown in the presence or absence of doxycycline, and Pak1-Rac interaction was examined by immnuoprecipitations. Overexpression of merlin inhibited the interaction between Pak1 and Rac, as demonstrated by the reduced levels of Rac coimmunoprecipitated with Pak1 and vice versa. The overall reduction in this interaction was approximately 4-fold in the presence of merlin (Figure 2A). While the levels of Rac1 were not altered in the presence or absence of merlin (Figure 2A), the levels of the GTP-bound form of Rac1 were decreased by approximately 2-fold in cells expressing merlin (Figure 2A). These reduced levels of Rac-GTP could explain in part the lower levels of Rac-Pak1 complexes in the presence of merlin.

We also examined the effect of merlin expression on the interactions of Pak1 with other adaptor proteins. This was done by immunoprecipitation and Western blot analysis of the relevant interactions. Merlin expression did not affect the interaction between Pak1 and NCK, Pak1 and β-pix, or Pak1 and p95PKL, as similar amounts of Pak1 and the various binding proteins were coimmunoprecipitated in the presence or absence of increased merlin expression (Figures 3B–3D). Merlin expression also did not alter the overall expression levels of these proteins (data not shown). In contrast, the interaction of Pak1 and paxillin was greatly reduced upon increased merlin expression (Figure 3A). When merlin was overexpressed in the RT4-67 cell lines, an average of 10-fold reduction in the Pak1-paxillin interaction was evident from the reduced levels of paxillin coimmunoprecipitated with Pak1 and vice versa. Again, expression of merlin did not affect the overall levels of paxillin or Pak1 expression in the cells (Figure 3A).

Based on these observations, we propose that merlin can inhibit Pak1 activation by binding directly to the Pak1 PBD and interfere with the binding of active Rac to the Pak1 PBD. Merlin might also function by directly reducing the level of active Rac1 that is available to bind and activate Pak1. Similarly, merlin could interfere directly with the interaction of Pak1 to paxillin, or this effect could be a consequence in the reduction of active Rac, which is required for the recruitment of Pak1 to focal adhesion complexes (Brown et al., 2002).

Loss of Merlin Results in Increased Pak Activity

We next tested the possibility that merlin expression might affect Pak1 activation. The phosphorylation status of Pak1 serves as a direct indication of the activation status of the kinase (Buchwald et al., 2001; Chong et al., 2001). To examine differences in the phosphorylation state of Pak1, we used 2-dimensional gel analysis to separate the different forms of activated Pak1 (Garcia Arguinzonis et al., 2002). MEFs were serum starved for 24 hr and then treated for 5 min with PDGF. Extracts were prepared from cells directly into sample buffer and quantified. Equal amounts of protein were separated by isoelectric focusing on an immobilized 4–7 pH gradient. Subsequently, the extracts were separated by SDS-PAGE in the second dimension, transferred to membranes, and analyzed by Western blotting using an anti-Pak1 antibody. As shown in Figure 4B, under conditions of serum starvation only two forms of Pak1 were detected in the extracts, most likely corresponding to non- or hypophosphorylated forms of Pak1. When extracts

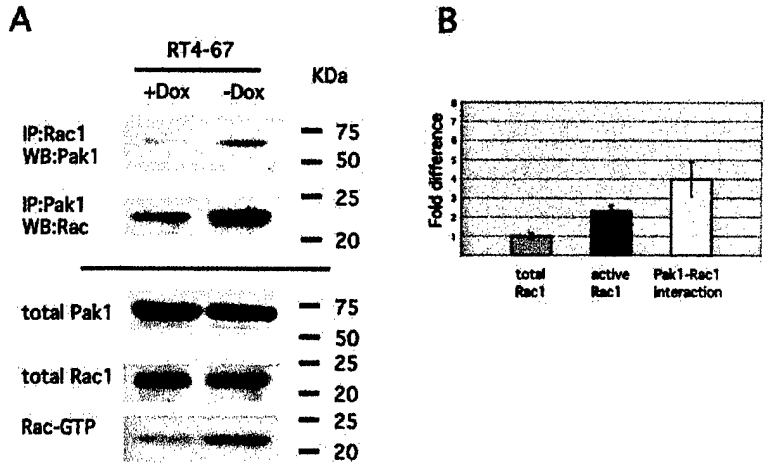

Figure 2. Merlin Reduces Levels of Rac-GTP and Interferes with Pak1-Rac Interactions

Western blot analysis of total protein levels and immunopreciptates from RT4-67 Schwann cells grown in the presence or absence of doxycycline. Pak1 and Rac1 (A). Quantification of the fold-differences in the levels of Rac1 and Rac1-GTP and in the interaction between Rac1 and Pak1 (B). The data represent the average of five independent experiments.

prepared from PDGF-treated MEFs were examined, however, several additional spots were detected. These spots correspond to hyperphosphorylated forms of Pak1, as they appear to be more acidic than the forms of Pak1 found in the serum-starved cells and separate in a signature pattern of a phospho-protein with multiple phosphorylation sites (Garcia Arguinzonis et al., 2002). This also correlated with the increased kinase activity of Pak1, as assessed directly by an in vitro kinase assay employing MBP (myelin basic protein) as a substrate (Figure 4A). To confirm that the additional Pak1 forms are due to phosphorylation, extracts of PDGF-treated cells were incubated with protein phosphatase 1 (PP1) in the presence or absence of protein phosphatase inhibitors. The protein phosphatase treatment resulted in the disappearance of the additional acidic forms of the protein that appear after the PDGF treatment (see Figure 4B), indicating that the additional spots appearing after PDGF stimulation are phosphorylated forms of Pak1. As expected, the inclusion of phosphatase inhibitors in the reaction prevented the loss of the phosphorylated species (data not shown).

To address the effect of merlin on Pak1 activation in vivo, the consequence of loss of merlin expression in MEFs was examined. Mouse embryo fibroblasts (MEFs) were prepared from animals carrying a conditional knockout (floxed) allele of *Nf2* ($Nf2^{flox2}$) (Giovannini et al., 2000). In addition to the $Nf2^{flox2}$ allele, the cells carried either a wild-type *Nf2* allele ($Nf2^{flox2/+}$) or a *Nf2* deletion allele ($Nf2^{flox2/-}$) (McClatchey et al., 1998). The MEFs were then infected with adenovirus expressing Cre-recombinase (ad-Cre), which led to the inactivation of the floxed *Nf2* allele. To test for loss of merlin expression in the ad-Cre treated $Nf2^{flox2/-}$ MEFs, extracts were prepared 96 hr after infection. One milligram of extract was used to immunoprecipitate merlin, and merlin levels were examined by Western blotting. The ad-Cre-treated $Nf2^{flox2/-}$ MEFs lacked detectable merlin protein 96 hr after infection, while merlin levels were not altered in ad-Cre $Nf2^{flox2/+}$ MEFs (Figure 5B). As a control, infection of the

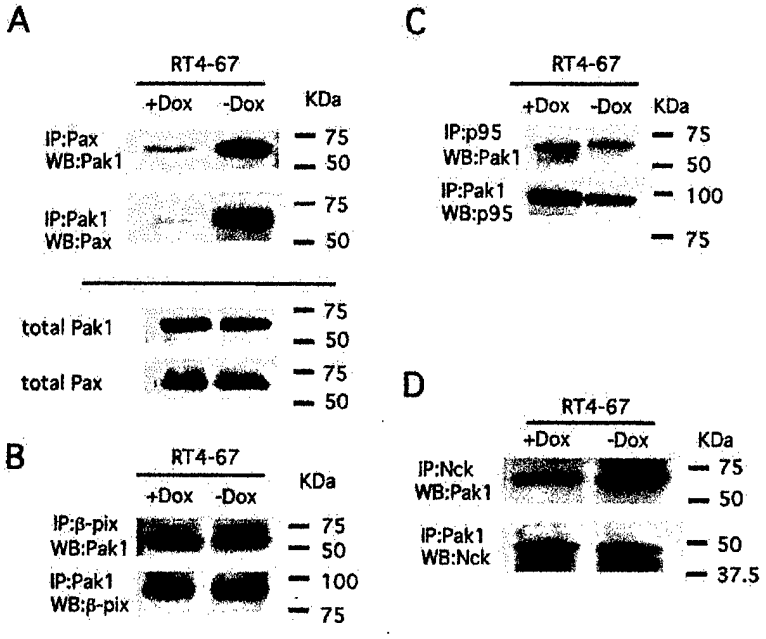

Figure 3. Merlin Interferes with Pak1-Paxillin Interactions

Western blot analysis of total protein levels and immunopreciptates from RT4-67 Schwann cells grown in the presence or absence of doxycycline: Pak1 and paxillin (A), Pak1 and β-pix (B), Pak1 and p95PKL (C), and Pak1 and Nck (D).

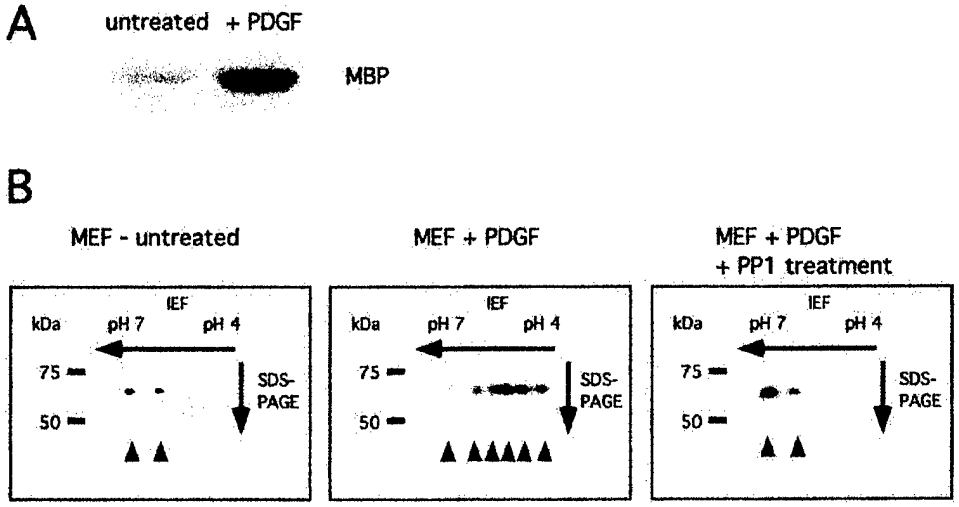

Figure 4. Analysis of Pak1 Phosphorylation by 2-Dimensional Gel Analysis

In vitro kinase assay of Pak1 immunoprecipitated from serum-starved or PDGF-treated (5 ng/ml) NIH3T3 cells, employing MBP as a substrate (A). Western blot analysis of Pak1 in MEFs that were serum starved (left), treated with PDGF (5 ng/ml) (center), and treated with protein phosphatase 1 (2.5 U/ml) (right).

cells with an adenovirus expressing the LacZ gene did not alter merlin levels (data not shown). The status of Pak1 was then analyzed in the MEFs. The ad-Cre-treated and adenovirus control-treated $Nf2^{flox2/-}$ and ad-Cre-treated $Nf2^{flox2/+}$ MEFs were plated at the same cellular densities and allowed to adhere to the tissue culture dish. The MEFs were then serum starved for 24 hr and extracted into sample buffer, and the status of Pak1 phosphorylation was analyzed by 2D gel analysis. Under conditions of serum starvation, Pak1 was not activated in control-treated MEFs, as demonstrated by detection of only hypophosphorylated forms of Pak1 (Figure 5A). However, in the ad-Cre treated $Nf2^{flox2/-}$ MEFs, which had lost the expression of merlin, a marked activation of Pak1 was observed, as indicated by the appearance of several phosphorylated forms of the kinase (Figure 5A). Thus, loss of merlin expression in MEFs promoted Pak1 activation under conditions normally associated with inactivity. These data are consistent with merlin functioning as an inhibitor of Pak1.

Merlin Expression Inhibits Pak Activation

As loss of merlin expression resulted in the appearance of activated forms of Pak1, we examined the possibility that the overexpression of merlin would inhibit Pak1 activation. In the RT4-67 Schwann cells basal levels of merlin are extremely low (Morrison et al., 2001). To assess Pak1 activity in these cells, RT4-67 cells were serum starved for 24 hr, protein was extracted, and the activation status of Pak1 was examined by 2D gel analysis. As shown in Figure 5C, the basal activity of Pak1 was relatively high in the RT4 cells. To assess whether the expression of merlin would affect the activity of Pak1 in these cells, the expression of merlin was induced by the addition of doxycycline into the growth media 48 hr prior to the harvesting of the cells, in the same manner as described above. Indeed, increased expression of merlin significantly reduced the levels of activated forms of Pak1 (Figure 5C). Thus, the reintroduction of merlin into the RT4 schwannoma cells, which display a high level of basal Pak1 activity, results in inhibition of Pak1 activation.

The data reported here are in agreement with previous work from us and others implicating merlin as a negative regulator of Rac-signaling. Specifically, overexpression of merlin has been shown to inhibit Rac-induced activation of c-Jun N-terminal kinase (JNK) and AP-1 transcription, while loss of merlin further resulted in elevated JNK basal activity and activation of AP-1. Loss of merlin also induced cytoskeleton changes that are phenotypically consistent with Rac activation, including membrane ruffling and increased cellular motility (Shaw et al., 2001). This is similar to the higher motility of cells expressing activated Rac alleles. While these data support a functional connection between merlin and Rac, they do not establish where in the Rac pathway merlin might act. Our data indicate that merlin can act both at the level of Rac activation and downstream of Rac, at the level of Pak activation.

The overexpression of Rac can induce transformation and anchorage-independent growth of cells (Khosravi-Far et al., 1995; Qiu et al., 1995). As Rac effectors, PAKs have been shown to mediate some of these signals. Using activated or dominant-negative forms of the proteins, the Paks have been shown to be involved in focal complex formation and membrane ruffling in various cell types (Daniels et al., 1998; Manser et al., 1997; Sells et al., 1997). Paks also have a role in signal transduction from Rac to JNK. Some reports have concluded that activated Pak1 or Pak3 can lead to upregulation of JNK activity; however, further studies are required to fully establish this connection (Brown et al., 1996; Zhang et al., 1995). In addition, recent data point to involvement of Pak in the regulation of the MAPK pathway. Pak can phosphorylate Raf-1 on serine 338 and induce phosphorylation of Mek1 on serine 298 (Diaz et al., 1997; Frost et al., 1997; King et al., 1998). Moreover, Rac or

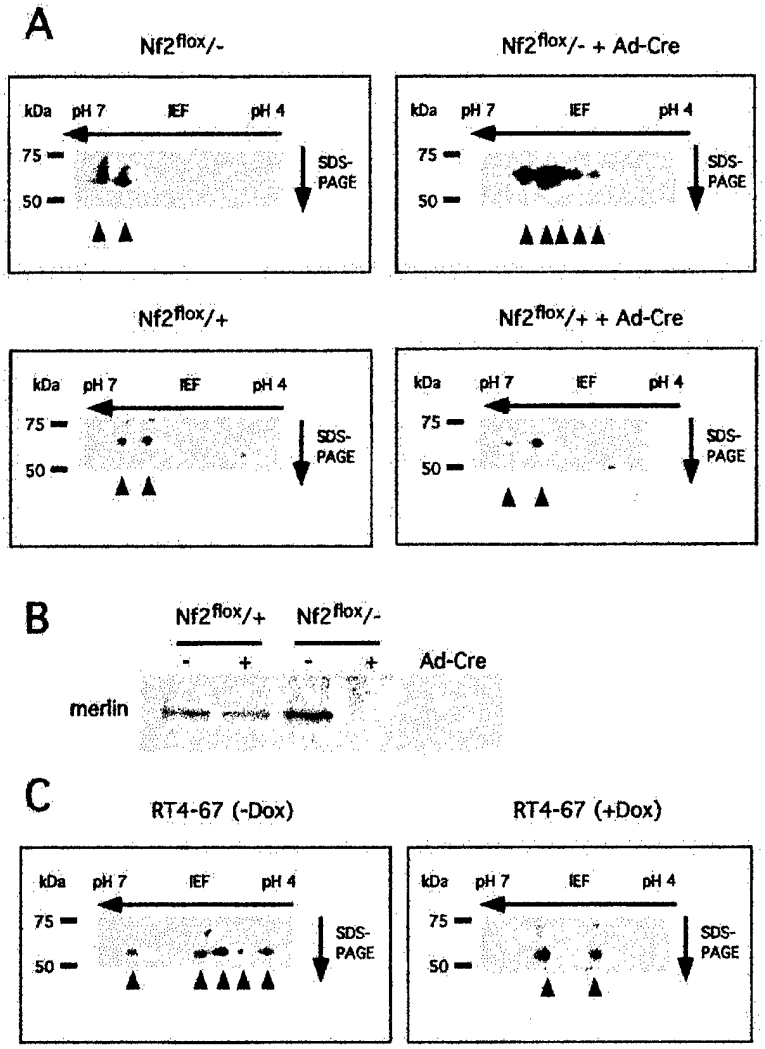

Figure 5. Analysis of Pak1 Phosphorylation by 2-Dimensional Gel Analysis

Western blot analysis of Pak1 in serum-starved untreated MEF clones that express merlin ($Nf2^{flox2/-}$, $Nf2^{flox2/+}$), serum-starved clones infected with adenovirus-Cre that express merlin ($Nf2^{flox2/+}$ + ad-Cre), and serum-starved clones infected with adenovirus-Cre that does not express merlin ($Nf2^{flox2/-}$ + ad-Cre). The various phosphorylated forms of Pak1 are indicated by arrows (A). IP-Western blot analysis of merlin expression in non-treated and ad-Cre-treated MEF lines (B). Western blot analysis of Pak1 in RT4 Schwann cells that are serum starved and either untreated (−Dox) or overexpressing merlin (+Dox). The various phosphorylated forms of Pak1 are indicated by arrows (C).

cdc42 has been reported to be required for the full activation of Ras by Raf. Pak may mediate the effect of Rac/cdc42 through phosphorylation of Raf (Li et al., 2001; Macara et al., 1996).

Merlin might exert its inhibitory function by several different mechanisms, which could be overlapping. As we have shown, expression of merlin in the RT-4 inducible cell line resulted in reduced levels of Rac-GTP, without affecting the levels of total Rac1. Merlin, interestingly, binds RhoGDI (Maeda et al., 1999) and therefore may link between RhoGDI and Rac1, thus stabilizing the inactive state of Rac1. Alternatively, merlin might act by affecting the activity of β-pix, which is a Rac GEF (Manser et al., 1998). We have also demonstrated here that merlin binds to the PBD domain of Pak1, and this interaction could well disrupt association with Rac and the subsequent activation of Pak1.

The localization of Pak is important in the regulation of its activity. Pak1 activation requires signals converging from both growth factor receptors and integrins. One possible site of physical integration of these pathways is the scaffold protein paxillin (Turner, 2000). Pak has been shown to bind to paxillin via the p95PKL-Cool/Pix protein complex (Turner et al., 1999). The recruitment of Pak1-PIX-p95PKL to paxillin is triggered by binding of Rac-GTP to Pak1 and activation of the adaptor function of Pak1 (Brown et al., 2002). We have demonstrated that merlin interferes with the Pak1-paxillin interaction, but does not affect the interaction between Pak1 and β-pix, p95PKL, or NCK. It is probable that NCK and p95/PKL-Cool/Pix target Pak to different subcellualr locations, where Pak is activated by different stimuli. Thus, it is possible that merlin can specifically inhibit the cellular fraction of Pak1 that is to be recruited to focal adhesion complexes. Interestingly, merlin has been demonstrated to bind to paxillin and to β-integrin (Fernandez-Valle et al., 2002; Obremski et al., 1998). Therefore, merlin may bind Pak while it is bound to paxillin and act to both release it from paxillin and inhibit its full activation by Rac. Alternatively, there could exist separate pools of merlin with distinct functions. One pool of merlin may function by binding to Pak1 prior to its association with paxillin and prevent the association. Another fraction of merlin, which is associated with paxillin, might have other, additional functions. The existence of pools of merlin with different functions can be demonstrated with the identification of merlin mutants impaired in their ability to bind Pak1 and the functional comparison of these

mutants with the mutants of merlin unable to bind paxillin (Fernandez-Valle et al., 2002). It is important to note that the localization and function of merlin is also likely to be affected by phosphorylation at serine 518 (Kissil et al., 2002; Shaw et al., 2001). The effects of different merlin mutants, including those affecting serine 518, on the interaction with and inhibition of Pak are currently being investigated.

The data described here, combined with the fact that Pak phosphorylates and, perhaps, inactivates merlin supports a "feedforward" signaling model (Kissil et al., 2002; Xiao et al., 2002). In such a model, merlin would function normally to downregulate Rac/cdc42-induced signaling. Once activated, Rac/cdc42 can stimulate Pak activity, which in turn would lead to merlin phosphorylation and relief from its inhibitory effect. Given the fact that Rac signaling is necessary, and in some cases sufficient, for transformation, it is possible that merlin's inhibition of Rac/cdc42-signaling represents its tumor suppressor function. Studies employing dominant-negative mutants have shown that Rac is required for cellular transformation by Ras (Khosravi-Far et al., 1995; Qiu et al., 1995; Ridley et al., 1992; Roux et al., 1997). Rac has also been shown to regulate cell motility and invasiveness (del Peso et al., 1997; Evers et al., 2000; Habets et al., 1994; Sahai et al., 2001). The fact that $Nf2^{+/-}$ mice develop highly metastatic tumors, which display loss of the wild-type Nf2 allele, is also consistent with a merlin-Rac functional connection (McClatchey et al., 1998).

The data presented here demonstrate a direct connection between merlin and the Rac-signaling pathways, via the inhibition of Pak. The work ascribes a biochemical function to a tumor suppressor with previously unknown function. In addition, it identifies an established tumor suppressor in the process of Pak1 regulation, possibly linking Pak deregulation to tumorigenesis. Understanding the regulation of merlin by Rac/cdc42 and merlin's impact on these signaling pathways could lead to a more complete understanding for the role of merlin in tumor formation. Once these interactions are fully elucidated, the use of specific inhibitors can be assessed as therapeutic modalities for tumors bearing mutations in NF2.

Experimental Procedures

Cell Culture Conditions and Transfections

The RT4 Schwann cells and MEFs were grown in DME, 10% fetal calf serum, and antibiotics. In cases where merlin expression was involved, expression was induced by addition of 1 μg/ml Doxycycline for 48 hr prior to the experiment. All transfections were done with Lipofectamine (Invitrogen). For the experiments where Pak1 activation was examined, cells were serum starved by growing them for 24 hr in serum-free DME and antibiotics. Pak1 activation was stimulated by adding PDGF-BB (Sigma) at 5 ng/ml for 5 min. In experiments where cells were in suspension, tissue culture plates were coated with poly-HEME (Sigma) as previously described (Folkman and Moscona, 1978). Cells were trypsinized, treated with soybean Trypsin inhibitor (Sigma), and placed back onto regular tissue culture dishes or the poly-HEME coated plates for 4 hr before harvesting.

Plasmids and Antibodies

Expression plasmids used for transfection are pCDNA3-Nf2 (Kissil et al., 2002), pCMV-Pak1 (Sells et al., 1997), and pCDNA3-β-pix (Hashimoto et al., 2001). Plasmids for the in vitro translation were constructed by PCR of the FERM domain (1–313), C-terminal half (342–595), F1-F2 (1–217), and F3 (218–313) (sequences available upon request) and subcloning of the fragments into pCDNA3. Antibodies used were: for merlin, SC-331; Pak1, sc-881 and sc-882; β-pix, sc-10932; Nck, sc-290; Rac1, sc-217 (Santa Cruz Biotechnology); anti-paxillin mAb, anti-Rac1 mAb and anti-p95PKL mAb (BD Transduction Laboratories), and anti-paxillin pAb (Chemicon International).

Immunoprecipitations, Kinase Assays, and Rac Activation Assays

Cells were plated at 7.5×10^5/10 cm dish and transfected the next day. 48 hr after transfection the cells were serum starved for 24 hr and extracted into extraction buffer (10 mM Tris-HCl [pH 7.6], 150 mM NaCl, 0.5% NP40, 0.1% deoxycholate, 1 mM $NaVO_4$, and protease inhibitors). Lysates were precleared for 1 hr, then incubated with the primary antibody for 3 hr at 4°C, and protein-A or protein-G beads were added for an additional 2 hr. Complexes were washed extensively with extraction buffer and separated by SDS-PAGE. Pak1 kinase assays were preformed as described (Kissil et al., 2002), except that Pak1 was immunoprecipitated from the extracts and MBP was added as substrate at 0.5 μg/ml. Quantification of active Rac was done employing the Rac activation assay kit, according to manufacturer's instructions (Upstate). Quantification of all Western blot experiments was done by densitometry analysis using NIH Image v.1.63 of scanned data from at least three independent experiments.

In Vitro Binding Assays

Full-length and truncation mutants of merlin were produced using the TnT kit (Promega) with Methionine S^{35}. GST-Pak1 and GST-Pak1(70-143) were produced in bacteria as described (Thiel et al., 2002). The in vitro binding assays were preformed by incubation of 30 μl of GST, GST-Pak1, or GST-Pak1(70-143) bound to gluthathione beads (0.5 mg/ml) with equal amounts of in vitro translated merlin-S^{35} (determined beforehand by running 5% of each reaction on SDS-PAGE and autoradiography) in reaction buffer (50 mM Tris-HCl [pH 7.5], 120 mM NaCl, 10 mM $MgCl_2$, 5% Glycerol, 1% Triton X-100) at 4°C for 3 hr and washed several times with the reaction buffer. The beads were then boiled, separated by SDS-PAGE, treated with Amplify (Amersham Pharmacia), dried, and exposed to film.

2-Dimensional Analysis of Pak1 Activity

Cells were harvested directly into sample buffer (9.8 M Urea, 2% CHAPS, 5 ml IPG buffer 4-7, DTT 15 mg/ml). Extracts were incubated on ice for 10 min and centrifuged 10 min, 14,000 × g, 4°C. Extracts (100 μg) were cup loaded onto 7 cm, pH 4–7 IPG strips (Amersham Pharmacia) and resolved at 50 mA/strip for 100V for 30 min, 200V for 30 min, 400V for 30 min, 1000V for 60 min, 3500V for 5 hr, and 500V to a total of 20,000V·hours using a IPGphor unit (Pharmacia biotech). Strips were then washed in wash solution (50 mM Tris-HCl [pH 8.8], 6 M Urea, 30% Glycerol, 2% SDS) supplemented with 20 mg/ml DTT for 10 min at RT and followed by a wash in wash buffer supplemented with 25 mg/ml iodoacetamide for 10 min. The strips were then loaded onto a standard SDS-PAGE, separated, and transferred to Immobilon (Millipore). The blots were then used in Western blot analysis. Equal loading of protein was determined by blotting with an actin antibody (Santa Cruz Biotechnology).

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